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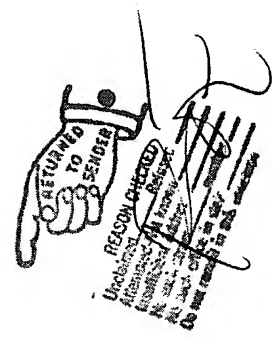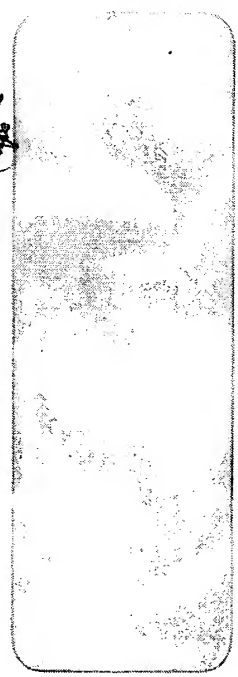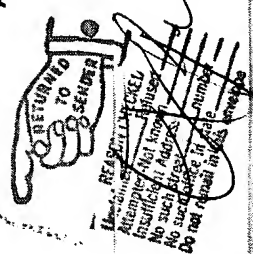
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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
10/005,220	12/04/2001	Keith D. Allen	R-741	6858

7590   03/08/2004

DELTAGEN, INC.
740 Bay Road
Redwood City, CA 94603

EXAMINER
PARAS JR, PETER

ART UNIT	PAPER NUMBER
1632	

DATE MAILED: 03/08/2004

Please find below and/or attached an Office communication concerning this application or proceeding.

	Application No.	Applicant(s)
Office Action Summary	10/005,220	ALLEN, KEITH D.
	Examiner	**Art Unit**
	Peter Paras, Jr.	1632

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE <u>3</u> MONTH(S) FROM
THE MAILING DATE OF THIS COMMUNICATION.
- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed
  after SIX (6) MONTHS from the mailing date of this communication.
- If the period for reply specified above is less than thirty (30) days, a reply within the statutory minimum of thirty (30) days will be considered timely.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133).
  Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any
  earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☐ Responsive to communication(s) filed on _____.

2a)☐ This action is **FINAL**.   2b)☒ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is
closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) *14 and 25-41* is/are pending in the application.

4a) Of the above claim(s) *39* is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☒ Claim(s) *14 and 25-41* is/are rejected.

7)☐ Claim(s) _____ is/are objected to.

8)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

9)☐ The specification is objected to by the Examiner.

10)☒ The drawing(s) filed on *04 December 2001* is/are: a)☒ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All   b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage
application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☒ Notice of References Cited (PTO-892)

2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)

3) ☒ Information Disclosure Statement(s) (PTO-1449 or PTO/SB/08)
Paper No(s)/Mail Date *1003*.

4) ☐ Interview Summary (PTO-413)
Paper No(s)/Mail Date _____.

5) ☐ Notice of Informal Patent Application (PTO-152)

6) ☐ Other: _____.

DETAILED ACTION

Applicant's preliminary amendment received on 12/3/02 has been entered.

Claims 1-13 and 15-24 have been cancelled. Claim 14 has been amended. New

claims 25-41 have been added. Claims 14 and 25-41 are pending.

Election/Restrictions

Restriction to one of the following inventions is required under 35 U.S.C. 121:

I.   Claims 14, 25-38 and 40-41, drawn to a transgenic mouse comprising a

disruption in an endogenous RPTPB gene, a method of producing the

same mouse, and cells comprising a disruption in an endogenous RPTPB,

classified in classes 800, 800, and 435, subclasses 18, 21 and 325.

II.   Claim 39, drawn to a targeting construct for an RPTPB gene, classified in

class 435, subclass 320.1.

Inventions I and II are unrelated. Inventions are unrelated if it can be shown that

they are not disclosed as capable of use together and they have different modes of

operation, different functions, or different effects (MPEP § 806.04, MPEP § 808.01). In

the instant case the different inventions are materially different products having different

functions. For example the transgenic mouse of Group I can be used as a disease

model while the targeting construct of Group II can be used for disrupting an RPTPB

gene in a cell *in vitro*. Because these inventions are distinct for the reasons given

above and have acquired a separate status in the art because of their recognized

divergent subject matter, different classification, and separate search requirement,

restriction for examination purposes as indicated is proper.

During a telephone conversation with Robert Driscoll on 2/10/04 a provisional

election was made without traverse to prosecute the invention of Group I, claims 14, 25-

38, and 40-41. Affirmation of this election must be made by applicant in replying to this

Office action. Claim 39 is withdrawn from further consideration by the examiner, 37

CFR 1.142(b), as being drawn to a non-elected invention.

Sequence Compliance

This application contains sequence disclosures that are encompassed by the

definitions for nucleotide and/or amino acid sequences set forth in 37CFR 1.821(a)(1)

and (a)(2). However, this application fails to comply with the requirements of 37 CFR

1.821 through 1.825 for the reason(s) set forth on the attached Notice To Comply With

Requirements For Patent Applications Containing Nucleotide Sequence And/Or Amino

Acid Sequence Disclosures.

Applicants are required to comply with all of the requirements of 37 C.F.R. §§

1.821 through 1.825. *Any* response to this Office Action, which fails to meet all of these

requirements, will be considered non-responsive. The nature of the noncompliance with

the requirements of 37 C.F.R. §§ 1.821 through 1.825 did not preclude the examination

of the application on the merits, the results of which are communicated below.

Drawings

The drawings filed on 12/4/01 are accepted.

Claim Objections

Claim 40 is objected to as it depends from a non-elected claim.

Claim Rejections - 35 USC § 101

35 U.S.C. 101 reads as follows:

Whoever invents or discovers any new and useful process, machine, manufacture, or composition of matter, or any new and useful improvement thereof, may obtain a patent therefor, subject to the conditions and requirements of this title.

Claim 40 is rejected under 35 U.S.C. 101 because the claimed invention is directed to non-statutory subject matter. The claim is directed to a murine embryonic stem cell comprising a disruption in a RPTPB gene the scope of which is interpreted to read on a murine embryonic stem cell *in vivo*, which embraces a mouse or rat comprising a naturally occurring disruption in an RPTPB gene. Amending the claim to read on an isolated murine stem cell may be sufficient to overcome the instant rejection.

Claims 14, 25-38, and 40-41 are rejected under 35 U.S.C. 101 because the claimed invention is not supported by either a substantial and specific asserted utility or a well-established utility.

The claims are directed to a transgenic mouse comprising a disruption in an endogenous RPTPB gene wherein the mouse exhibits embryonic lethality, wherein lethality occurs at E9.5-10.5 and the embryo exhibits reduced vascular development

The instant specification has contemplated that the nucleotide sequence set forth in SEQ ID NO: 1 encodes a RPTPB gene. The instant specification has further contemplated that disruption of the nucleotide sequence set forth in SEQ ID NO: 1 in a mouse will produce a phenotype related to RPTPB. The instant specification has purported that such mice may be used to identify agents that modulate or ameliorate a phenotype associated with a disruption in SEQ ID NO: 1.

The instant specification has disclosed a heterozygous transgenic mouse whose genome comprises a disruption in SEQ ID NO: 1 [that does not exhibit a phenotype], wherein a mouse embryo whose genome comprises a homozygous disruption in SEQ ID NO: 1 exhibits embryonic lethality, wherein lethality occurs at E9.5-10.5 and the embryo exhibits reduced vascular development. The claims embrace such a mouse and a method of making the mouse. The instant specification has discussed that phenotype, such as embryonic lethality exhibited by such a transgenic mouse could correlate to a disease or disorder. However, the evidence of record does not provide a correlation between the observed embryonic lethality and any disease or disorder. Moreover, while the specification has purported that the nucleotide sequence set forth in SEQ ID NO: 1 encodes a RPTPB, the evidence of record has failed to provide a correlation between any RBTPB related disease/disorder and embryonic lethality. The specification has provided general assertions that the claimed transgenic mice may be used to identify agents that affect a phenotype related to the mice.

As such, the asserted utility, for the transgenic mouse embraced by the claims, of screening agents that may affect a phenotype of said mouse as provided by the instant

specification and encompassed by the claims, does not appear to be specific and

substantial. The asserted utility does not appear specific and substantial to the skilled

artisan since the evidence of record has not provided any suggestion of a correlation

between any RPTPB, embryonic lethality, and any disease or disorder. Since the

evidence of record has not provided a correlation between embryonic lethality and any

disease or disorder, the utility of identifying agents that affect embryonic lethality is not

apparent. The evidence of record has not provided any other utilities for the transgenic

mouse embraced by the claims that are specific, substantial, and credible.

The asserted utility of the transgenic mouse embraced by the claims is based on

the expectation that disrupting the nucleotide sequence set forth in SEQ ID NO: 1 would

result in a detectable phenotype in the mouse. The phenotype observed in the

transgenic mice embraced by the claims is embryonic lethality. While the phenotypes

exhibited by the claimed transgenic mouse are contemplated to be associated with a

disease, the association of embryonic lethality with any disease has yet to be

elucidated. In fact the art suggests that phenotypes, such as embryonic lethality, are

greatly influenced by the genetic background of the transgenic knockout mouse. For

example, Casademunt et al report (EMBO, 1999, 18(21): 6050-6061) nrif -/- mice

exhibit differences in embryonic lethality that appear dependent on the genetic

background of the mouse. In short, transgenic nrif -/- mice in a BL6 background cannot

survive past E12 while in an Sv129 background such transgenic mice are viable and

healthy to adulthood. See the abstract and throughout the entire document.

Furthermore, LeCouter et al (Development, 1998, 125: 4669-4679) observe that a null

mutation in a p130 is lethal at E11-13 on a Balb/cJ background but when such a

mutation is crossed onto a C57BL/6 background the resulting mice are viable and

fertile. See the abstract and throughout the entire document. More to the point,

Harroch et al (Molecular and Cellular Biology, 2000, 20(20): 7706-7715; IDS) discuss

that an RPTPB null mutation crossed into a Swiss Webster genetic background result in

mice having no obvious abnormalities. See the abstract and throughout the entire

document. The instant specification has taught use of ES cells form a 129/OlaHSD

genetic background to generate chimeric mice, which were then bred onto a C57BL6

genetic background to produce the instantly claimed mice exhibiting embryonic lethality

as homozygous embryos. See pages 50-51 of the instant specification.

Therefore, the references suggest a need to provide independent evidence of an

association of embryonic lethality with a disease or disorder. However, neither the

specification nor any art of record provides evidence of the existence of a correlation

between embryonic lethality and a disease or disorder, leaving the skilled artisan to

speculate and investigate the uses of the transgenic mouse embraced by the claims.

The specification essentially gives an invitation to experiment wherein the artisan is

invited to elaborate a functional use for the transgenic mouse embraced by the claims.

In light of the above, the skilled artisan would not find the asserted utility of the

transgenic mouse embraced by the claims to be specific and substantial.

Claim Rejections - 35 USC § 112, 1st paragraph

The following is a quotation of the first paragraph of 35 U.S.C. 112:

> The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same and shall set forth the best mode contemplated by the inventor of carrying out his invention.

Claims 14, 25-38, and 40-41 are also rejected under 35 U.S.C. 112, first paragraph. Specifically, since the claimed invention is not supported by either a substantial and specific asserted utility or a well established utility for the reasons set forth above, one skilled in the art clearly would not know how to use the claimed invention.

In addition the following additional rejections are necessitated under 35 U.S.C. 112, first paragraph:

Both the specification and the state of the art have taught that the transgenic knockout technology requires the use of embryonic stem cells that have been genetically manipulated to comprise a disruption in a nucleotide sequence of interest. The specification has not taught creation of a transgenic knockout mouse by methods that do not require embryonic stem cells. Presently, the transgenic knockout technology is limited to the mouse system. See below.

With regard to the claim breadth, claims 38 and 40 embrace murine embryonic stem cells, which encompass species other than mice, such as rats and gerbils. The specification fails to teach use of embryonic stems from species other than mice. It is well known in the knockout art that the production of knockout animals other than mice is undeveloped. This is because ES cell technology is generally limited to the mouse

system, at present, and that only "putative" ES cells exist for other species. See

Moreadith et al. at page 214, Summary. Seamark (Reproductive Fertility and

Development, 1994) supports this observation by reporting that totipotency for ES cell

technology in many livestock species has not been demonstrated (page 6, Abstract).

Likewise, Mullins et al (Journal of Clinical Investigation, 1996) state, "although to date

chimeric animals have been generated from several species including the pig, in no

species other than the mouse has germline transmission of an ES cell been

successfully demonstrated." (page S38, column 1, first paragraph). Furthermore, claims

25-38 as written do not appear to require germline transmission of the disrupted

nucleotide sequence. These claims may be broadly interpreted to read on a single cell

comprising a disrupted nucleotide sequence. Since the claims do not require germline

transmission of the disrupted nucleotide sequence it would be unpredictable if an ES

cell comprises the disrupted nucleotide sequence. The evidence of record does not

support germline transmission of non-ES cells. Also, it would be unpredictable if a

disruption of a nucleotide sequence in a single cell would result in a phenotype; the

instant specification has not provided any uses for a transgenic mouse that does not

exhibit a phenotype resulting from disruption of a nucleotide sequence (see below).

Amending the claims to read on a "transgenic mouse whose genome comprises" would

be sufficient to suggest germline transmission. Given the unpredictable state of the art

it would have required undue experimentation for the skilled artisan to create transgenic

knockout non-human animals of species other than the mouse.

Claims 14 and 25-27 encompasses transgenic mice that comprise a disruption in

a RPTPB gene, particularly the nucleotide sequence set forth in SEQ ID NO: 1, that do

not exhibit any particular phenotype. Claims 14 and 28-38 embrace transgenic mice

exhibiting a particular phenotype, wherein a broad interpretation of the claimed animals

could read on disruption of a RPTPB gene in a single cell. Claims 14, 25 and 27-38

embrace transgenic mice comprising a heterozygous disruption of an RPTPB gene.

The specification has taught that transgenic mouse embryos whose genomes comprise

a homozygous disruption of the RPTPB gene exhibit a phenotype of embryonic lethality.

The specification has not provided guidance correlating to a phenotype in the other

transgenic mice embraced by the claims. The state of the art at the time of filing was

such that one of skill could not predict the phenotype of a knockout mouse (Moreadith et

al., 1997, J. Mol. Med., Vol. 75, pages 208-216; see page 208, column 2, last full

paragraph). Also see Leonard et al (Immunological Reviews, 1995, pages 97-114) who

discuss that inactivation of the gene encoding cytokine receptor γ chain in transgenic

mice results in a phenotype different from that expected. Finally, Moens et al.

(Development, Vol. 119, pages 485-499, 1993) disclose that two mutations produced by

homologous recombination in two different locations of the N-myc gene produce two

different phenotypes in mouse embryonic stem cells, one leaky and one null (see

abstract). The specification has asserted that the nucleotide sequence set forth in SEQ

ID NO: 1 encodes a RPTPB. However, it would be difficult to predict any phenotype

resulting from disruption of the sequence of SEQ ID NO: 1 in light of the above.

Moreover, as the claims read on disruption of a RPTPB gene in a single cell, it would be

unpredictable if such a disruption would result in any phenotype. The specification discloses a phenotype exhibited by transgenic mouse embryos whose genome comprises a homozygous disruption in the nucleotide sequence set forth in SEQ ID NO: 1 is embryonic lethality. See pages 50-52 of the specification. Claims 14 and 25-27 as written, do not include a phenotype that differs from the wild-type mouse. One of skill in the art would not know how to use a transgenic knockout non-human animal that lacks a phenotype, particularly because the instant specification has not provided uses for such; the transgenic mice that have a phenotype may be used for drug testing according to the instant specification. The claims (14, 25 and 27-38) embracing heterozygous mice are not enabled as the specification has not disclosed a phenotype for such and because as previously stated, the skilled artisan would not know how to use a transgenic mouse lacking a phenotype. Accordingly, the claims are not commensurate in scope with the phenotype disclosed in the specification. Given the unpredictable nature of a phenotype that results from disruption of a nucleotide sequence it would have required undue experimentation for the skilled artisan to make and use the invention as claimed.

It is noted that the claims, which embrace a homozygous disruption of the RPTPB gene, are directed to a transgenic mouse. However, use of transgenic mouse language implies a full term mouse was created. This language appears inconsistent with the teachings of the specification because it appears that the homozygous mouse embryos did not mature to term given the phenotype of embryonic lethality.

Claim Rejections - 35 USC § 102

The following is a quotation of the appropriate paragraphs of 35 U.S.C. 102 that

form the basis for the rejections under this section made in this Office action:

A person shall be entitled to a patent unless –

(a) the invention was known or used by others in this country, or patented or described in a printed
publication in this or a foreign country, before the invention thereof by the applicant for a patent.

Claims 14 and 25-27 are rejected under 35 U.S.C. 102(a) as being anticipated by

Harroch (IDS).

The claims are directed to a transgenic mouse comprising a disruption in an

RPTPB gene.

Harroch et al teach transgenic mice comprising a homozygous disruption in the

RPTPB gene.

Accordingly, Harroch et al anticipate all of the instant claim limitations.

Conclusion

No claim is allowed.

Any inquiry concerning this communication or earlier communications from the examiner(s) should be directed to Peter Paras, Jr., whose telephone number is (571) 272-0732. The examiner can normally be reached Monday-Friday from 8:30 to 4:30 (Eastern time).
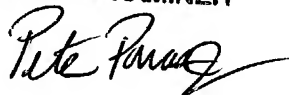
If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Amy Nelson, can be reached at 571-272-0804. Papers related to this application may be submitted by facsimile transmission. Papers should be faxed via the PTO Fax Center located in Crystal Mall 1. The faxing of such papers must conform with the notice published in the Official Gazette, 1096 OG 30 (November 15, 1989). The CM1 Official Fax Center number is (703) 872-9306.

Inquiries of a general nature or relating to the status of the application should be directed to Dianiece Jacobs whose telephone number is (571) 272-0532.

Peter Paras, Jr.

Art Unit 1632

PETER PARAS, JR.
PRIMARY EXAMINER

NOTICE TO COMPLY WITH REQUIREMENTS FOR PATENT APPLICATIONS CONTAINING NUCLEOTIDE SEQUENCE AND/OR AMINO ACID SEQUENCE DISCLOSURES

The nucleotide and/or amino acid sequence disclosure contained in this application does not comply with the requirements for such a disclosure as set forth in 37 C.F.R. 1.821 - 1.825 for the following reason(s):

[X] 1. This application clearly fails to comply with the requirements of 37 C.F.R. 1.821-1.825. Applicant's attention is directed to these regulations, published at 1114 OG 29, May 15, 1990 and at 55 FR 18230, May 1, 1990.

[ ] 2. This application does not contain, as a separate part of the disclosure on paper copy, a "Sequence Listing" as required by 37 C.F.R. 1.821(c).

[ ] 3. A copy of the "Sequence Listing" in computer readable form has not been submitted as required by 37 C.F.R. 1.821(e).

[ ] 4. A copy of the "Sequence Listing" in computer readable form has been submitted. However, the content of the computer readable form does not comply with the requirements of 37 C.F.R. 1.822 and/or 1.823, as indicated on the attached copy of the marked -up "Raw Sequence Listing."

[ ] 5. The computer readable form that has been filed with this application has been found to be damaged and/or unreadable as indicated on the attached CRF Diskette Problem Report. A Substitute computer readable form must be submitted as required by 37 C.F.R. 1.825(d).

[ ] 6. The paper copy of the "Sequence Listing" is not the same as the computer readable from of the "Sequence Listing" as required by 37 C.F.R. 1.821(e).

[X] 7. Other: Figure 2A contains an unidentified sequence.

Applicant Must Provide:

[X] Either a substitute Figure 2A that identifies the sequence or an amendment to the specification in the Drawings section that identifies the sequence in Figure 2A.

[ ] An initial or substitute paper copy of the "Sequence Listing", as well as an amendment directing its entry into the specification.

[ ] A statement that the content of the paper and computer readable copies are the same and, where applicable, include no new matter, as required by 37 C.F.R. 1.821(e) or 1.821(f) or 1.821(g) or 1.825(b) or 1.825(d).

For questions regarding compliance to these requirements, please contact:
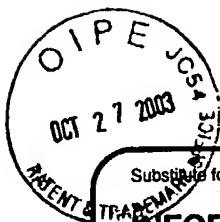
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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(use as many sheets as necessary)

Complete if Known	
Applicatin Numbr	10/005,220
Filing Date	December 4, 2001
First Named Inventr	ALLEN
Art Unit	Unassigned
Examiner Name	Unassigned
Attorney Docket Number	R-741

| Sheet | 2 | of | 2 | | |

OTHER PRIOR ART -- NON PATENT LITERATURE DOCUMENTS			
Examiner Initials *	Cite No.[1]	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T[2]
PP	AA	S. HARROCH, et al., "No Obvious Abnormality in Mice Deficient in Receptor Protein Tyrosine Phosphatase β" *Molecular and Cellular Biology*, Vol. 20**(20)** October 2000, pp.7706-7715	
PP	AB	TAKAFUMI SHINTANI, et al., "Neurons and well as astrocytes express proteoglycan-type protein tyrosine phosphatase ζRPTPβ: analysis of mice in which the *PTPζRPTPβ* gene was replaced with the *LacZ* gene" Vol. 247, (1998) pp. 135-138	

Examiner Signature	*Pete Parasg*	Date Considered	*3/3/04*

→

PTO/SB/08B (10-01)
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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(use as many sheets as necessary)

Complete if Known			
Application Number	10/005,220		
Filing Date	December 4, 2001		
First Named Inventor	ALLEN		
Art Unit	Unassigned		
Examiner Name	Unassigned		

Sheet	2	of	2	Attorney Docket Number	R-741

FOREIGN PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Foreign Patent Document			Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear	T[6]
		Country Code[3]	Number[4]	Kind Code[5] (if known)				
P̃P	AC	WO	00/06712	A2	02/10/00	Merck Frosst Ca. Inc.	————	

Examiner Signature	*Pete Parag*	Date Considered	3/8/04

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

[1] Applicant's unique citation designation number (optional). [2] Applicant is to place a check mark here if English language Translation is attached.

Burden Hour Statement: This form is estimated to take 2.0 hours to complete. Time will vary depending upon the needs of the individual case. Any comments on the amount of time you are required to complete this form should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, Washington, DC 20231. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Assistant Commissioner for Patents, Washington, DC 20231.
Unsaved Document

		Application/Control No.	Applicant(s)/Patent Under Reexamination
Notice of References Cited		10/005,220	ALLEN, KEITH D.
		Examiner	Art Unit	Page 1 of 2
		Peter Paras, Jr.	1632	

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	Classification
	A	US-			
	B	US-			
	C	US-			
	D	US-			
	E	US-			
	F	US-			
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	I	US-			
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	L	US-			
	M	US-			

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	N					
	O					
	P					
	Q					
	R					
	S					
	T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	Casademunt et al. EMBO, 1999, 18(21): 6050-6061.
	V	LeCouter et al. Development, 1998, 125: 4669-4679.
	W	Leonard et al. Immunological Reviews. 1995, 97-114.
	X	Moens et al. Development. 1993, 119: 485-499.

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

		Application/Control No.	Applicant(s)/Patent Under Reexamination
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	A	US-			
	B	US-			
	C	US-			
	D	US-			
	E	US-			
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FOREIGN PATENT DOCUMENTS

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NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	Moreadith et al. J. Mol. Med., 1997, 75: 208-216.
	V	Mullins et al. J. Clin. Invest., 1996, 98(11): S37-40.
	W	Seamark et al. Reprod. Fertil Dev., 1994, 6(5): 653-657
	X	

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The zinc finger protein NRIF interacts with the neurotrophin receptor p75^{NTR} and participates in programmed cell death

Elisabeth Casademunt, Bruce D.Carter[1], Isabel Benzel, José M.Frade[2], Georg Dechant and Yves-Alain Barde[3]

Department of Neurobiochemistry, Max Planck Institute of Neurobiology, D-82152 Martinsried, Germany and [1]Center for Molecular Neuroscience, Vanderbilt University Medical School, Nashville, TN 37232, USA

[2]Present address: Instituto Cajal de Neurobiología, CSIC, Avda. Dr. Arce 37, E-28002 Madrid, Spain

[3]Corresponding author
e-mail: yves_barde@neuro.mpg.de

E.Casademunt and B.D.Carter contributed equally to this work

NRIF (neurotrophin receptor interacting factor) is a ubiquitously expressed zinc finger protein of the Krüppel family which interacts with the neurotrophin receptor p75^{NTR}. The interaction was first detected in yeast and then biochemically confirmed using recombinant GST–NRIF fusions and p75^{NTR} expressed by eukaryotic cells. Transgenic mice carrying a deletion in the exon encoding the p75^{NTR}-binding domain of NRIF display a phenotype which is strongly dependent upon genetic background. While at the F$_2$ generation there is only limited (20%) embryonic lethality, in a congenic BL6 strain *nrif*$^{-/-}$ mice cannot survive beyond E12, but are viable and healthy to adulthood in the Sv129 background. The involvement of NRIF in p75^{NTR}/NGF-mediated developmental cell death was examined in the mouse embryonic neural retina. Disruption of the *nrif* gene leads to a reduction in cell death which is quantitatively indistinguishable from that observed in *p75*$^{NTR-/-}$ and *ngf*$^{-/-}$ mice. These results indicate that NRIF is an intracellular p75^{NTR}-binding protein transducing cell death signals during development.
Keywords: apoptosis/embryonic lethality/genetic background/p75^{NTR}/zinc finger protein

Introduction

The neurotrophins, including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 and neurotrophin-4/5 have a well-established and essential role as agents preventing programmed cell death during the development of the mammalian nervous system (Snider, 1994). This function is mediated through the binding of neurotrophins to a family of tyrosine kinases, the trk receptors. In addition, there is a considerable body of evidence to suggest that the neurotrophins have a wider role in regulating neuronal function, including the modulation of synaptic plasticity (McAllister et al., 1999), the regulation of dendritic arborization (Ruit et al., 1990;

McAllister et al., 1996) or the control of growth cone guidance in vitro (Zheng et al., 1994; Gallo et al., 1997). Recently, and somewhat surprisingly, the neurotrophins have also been shown to promote programmed cell death during normal development. In contrast to the survival activity mediated via the trk receptors, neurotrophin-induced apoptosis is a function mediated by a neurotrophin receptor unrelated to the trks, the p75^{NTR} receptor (Dechant and Barde, 1997). A role for p75^{NTR} in cell death has been documented (for a review, see Casaccia-Bonnefil et al., 1999) using cultured cells (Rabizadeh et al., 1993; Casaccia-Bonnefil et al., 1996; Gu et al., 1999; Soilu-Hänninen et al., 1999), including neurons (Barrett and Bartlett, 1994; Davey and Davies, 1998). In vivo, p75^{NTR}-mediated cell death has been observed in the developing avian and murine retinae (Frade et al., 1996; Frade and Barde, 1999) and in sympathetic neurons (Bamji et al., 1998). However, the actual binding partners of p75^{NTR} involved in pro-apoptotic signalling are not well characterized.

Initially, p75^{NTR} was thought merely to modulate neurotrophin binding to the trks and the subsequent signal transduction through well-established tyrosine kinase receptor pathways (Segal and Greenberg, 1996). Indeed, substantial evidence supports such a co-receptor role for p75^{NTR}, including an increase in binding affinity (Hempstead et al., 1991; Battleman et al., 1993) and selectivity (Benedetti et al., 1993) of a given trk for its cognate neurotrophin. Also, p75^{NTR} associates with all three trk receptors and can be co-immunoprecipitated with them (Bibel et al., 1999). In addition, trk signalling is enhanced in the presence of p75^{NTR} (Davies et al., 1993; Lee et al., 1994; Verdi et al., 1994). However, the widespread expression of p75^{NTR}, including in a variety of cells not expressing catalytic forms of trks, suggested that p75^{NTR} also has an independent function. The first indication that p75^{NTR} could initiate a signal transduction pathway independently of the trks was the observation that neurotrophin binding to p75^{NTR} stimulated the hydrolysis of sphingomyelin to produce the intracellular mediator ceramide (Dobrowsky et al., 1995), although the function of this lipid second messenger is still not fully understood. Further, NGF was reported to activate the transcription factor NF-κB in neuroblastoma cells (Korner et al., 1994) as well as in primary cultures of sensory (Wood, 1995) and sympathetic neurons (Maggirwar et al., 1998), and in both Schwann cells (Carter et al., 1996; Khursigara et al., 1999) and oligodendrocytes (Ladiwala et al., 1998; Yoon et al., 1998). This activation of NF-κB was shown to occur through binding to the p75^{NTR} receptor (Carter et al., 1996; Khursigara et al., 1999). Neurotrophin binding to p75^{NTR} has also been observed to stimulate the stress-activated kinase, c-jun kinase, which has been implicated in mediating p75^{NTR}-induced apoptosis
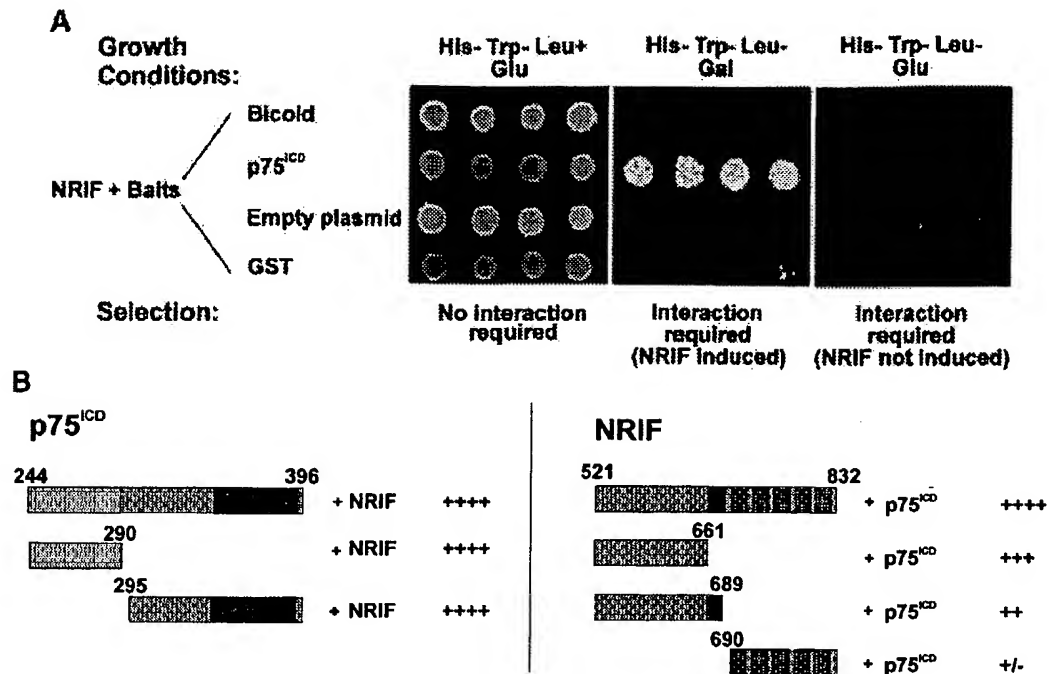
Fig. 1. (A) Isolation of NRIF as a p75^{NTR} interactor from a yeast two-hybrid screen. Screening of a human fetal library for p75^{NTR}-interacting proteins resulted in the isolation of several candidate cDNAs. Yeast clones capable of growing under selective conditions (His⁻ Trp⁻ Leu⁻ on galactose plates) were tested further against other baits (bicoid, GST) and empty plasmid to confirm the specificity of the interaction. Only yeast expressing both p75ICD and NRIF grew under selective conditions. (B) Mapping of the interacting regions. Deletion constructs expressing the indicated fragments of p75ICD (left) and mouse NRIF (right) were co-transfected in yeast and their ability to support growth on selective media was compared with yeast co-expressing p75ICD and NRIF (residues 521–832). Whereas the three p75ICD fragments supported growth at comparable rates (left), the NRIF region N-terminal to the zinc fingers was clearly the most important for the interaction (right).

(Casaccia-Bonnefil et al., 1996; Bamji et al., 1998; Yoon et al., 1998).

The p75^{NTR} receptor belongs to a large family of transmembrane proteins including the tumour necrosis factor (TNF) receptors R1 and R2, Fas, CD40, CD30, OX40, lymphotoxin-β receptor, CD27 and DR3, 4 and 5 (for a review, see Casaccia-Bonnefil et al., 1998). Cysteine repeats in the extracellular domains originally formed the basis for defining members of this family. In addition, the cytoplasmic domains of several of these receptors have features in common, suggesting their involvement in protein–protein interactions. At the structural level, the cytoplasmic domain of both FAS/Apo-1/CD95 and of p75^{NTR} shows a similar motif consisting of a closely packed bundle of six short α-helices (Huang et al., 1996; Liepinsh et al., 1997). Several of these protein–protein interaction domains are now known to initiate signalling cascades leading to cell death or to the activation of the transcription factor NF-κB. For receptors such as TNFR1 and R2, as well as Fas, CD40 or CD30, a number of cytoplasmic proteins have been identified which associate with these domains and appear to function as signal transducers (for a review, see Nagata, 1997). One such adaptor protein, TRAF6, initially identified on the basis of its interactions with CD40 and interleukin-1 (IL-1) (see Arch et al., 1998), has been implicated as mediating p75^{NTR} activation of NF-κB (Khursigara et al., 1999).

We report here the identification of a novel protein which interacts with the intracellular domain of p75^{NTR} which we term NRIF, for Neurotrophin Receptor Interacting Factor. NRIF appears to play a role in p75^{NTR}-

mediated apoptosis since mice with the nrif gene deleted exhibit reduced cell death in early retinal cells, similar to p75$^{-/-}$ and the ngf$^{-/-}$ mice (Frade and Barde, 1999).

Results

Isolation of NRIF as an intracellular interactor of p75^{NTR} in a yeast two-hybrid screen

To identify proteins interacting with p75^{NTR}, the intracellular domain (ICD) of the rat p75^{NTR} receptor (residues 244–396) was fused to the C-terminus of the lexA DNA-binding protein in a yeast expression vector (Gyuris et al., 1993). This bait plasmid was co-transfected into a leucine-deficient yeast strain containing the lexA-binding domain upstream of the LEU2 gene, along with a human fetal brain library fused to the B42 transcription activator under the control of a GAL1-inducible promoter in pJG4-5. We isolated 17 clones which demonstrated selective growth on galactose.

Upon sequence analysis, one of the clones, designated pJG-NRIF, was found to encode 1.3 kb of a novel sequence fused in the reading frame of B42 and having homology with a number of zinc finger proteins. This clone was also able to activate transcription of lacZ from the reporter plasmid pSH18-34 (which contains lexA-binding domains upstream of lacZ) when co-transfected with p75^{NTR}ICD, as evidenced by β-galactosidase staining (data not shown). To assess further the specificity of the interaction with p75^{NTR}, several 'false baits' were tested, including plasmids coding for the Drosophila protein bicoid, GST and the empty bait plasmid. Only co-transfection of

```
                    ↓  ↓                          KRAB A
  1   MEDSMASTLP  TTWPHESVKF  EDVSLTFTEE  EWAQLDFQQK  CLYREIMMEN
                            KRAB B
 51   YSNMISVEHH  FSKPNVISQL  EKAEDCWPMQ  REIPQDTLPE  CSWPSPDPGM

101   NSFPSKSPLM  KIEVVEVLTL  NKDVAGPRNA  LIQSLYPEDL  NPGNLKPAQL

151   PSKRLTDTEA  SRQKFRHFQY  EESAGPQKAM  SQLRKLCHQW  LQPWTRSKKQ

201   ILELLVLEQF  LNALPEKFRV  WVESQHPEDC  KAVVALLENM  TSVSKDDASL

251   ACSSEATDQL  KEKRKGVATL  PVTFAAEVPA  EEPVTFQDVA  VDFNEEEWRL

301   LGPTQKTEYH  DVMLETLGNL  VSVGWEPTLG  NRELTPDSPI  PVVKPIHDPN

351   TKDLSRNGTQ  STVFESILED  GVKEMHTIES  NQVGNLQEKG  HPQKKFSESS

401   KSQDQTSRHK  SQGSLNEVLP  RKYVKVKQKG  TGKRKGRTNT  ISMTRGLRIR

451   KQQKDSVENQ  GRSGSTPVTH  GSSIKKQQQG  SEQGKPGTSR  DPITLTVPAK

501   VYQKATGSKE  SILMDSSDAM  VSDVPPKIHQ  KGPEWHKVGE  SNNSMLQGSS

551   VQNHQMESGA  GRASDNSLLT  HALPVKSHQK  GYKEGNVQGN  RNSWKHIKPH

601   QKGSKGERVE  ELSTSEKHVP  YVKNHLKTSE  RGKDREINAS  IKCDPYIKTY

651   YRGSDVGRLR  RANNCRKAFS  LHAQQISFIK  IEKGSQVCHC  SECGKLFRNA

701   RYFSVHKKIH  TGERPYMCMA  CGKAFVQSSS  LTQBLRIEBG  ERPFECSECG

751   RTFNDRSAIS  QBLRTETGAK  PYHCERCGKA  FRQSSHLTRE  ERTETGERPY

801   VCIKCGKAFT  QSSHLIGHQK  THGIKFKKQP  KL*
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Fig. 2. Amino acid sequence of the predicted NRIF protein. Predicted amino acid sequence as translated from the *nrif* 6.3 cDNA clone (see text). Large arrows indicate the two potential translation initiation sites. The positions of the two N-terminal KRAB boxes, KRAB A and KRAB B, the pseudo-finger and the five zinc finger domains, are indicated. A group of regularly spaced leucine residues clustered at the N-terminal part of the protein, downstream of the KRAB domains, indicated in bold (L), could function as a nucleocytoplasmic signal (Nigg, 1997). The underlined sequence (residues 329–374) shares relatively high homology with several other KRAB-containing zinc finger proteins. The small arrow at position 530 marks the N-terminal end of the partial, human *nrif* cDNA isolated from the yeast two-hybrid screen. A potential nuclear localization signal is underlined. The sequence contains consensus sites for amidation, cAMP phosphorylation, CK2 phosphorylation, PK-C phosphorylation, myristolation and Asn glycosylation. Sequence data have been submitted to the DDBJ/EMBL/GenBank database under accession No. AJ242914.

pJG-NRIF with p75^{NTR}ICD enabled the yeast to survive (Figure 1).

### Cloning and sequence analysis of mouse NRIF

To obtain a full-length clone of mouse NRIF, a post-natal day 1 mouse cDNA library was screened with a 0.9 kb fragment from the human clone which contained the putative zinc finger domain. A 3.289 kb clone (clone 6.3) was isolated which contained a 2.645 kb uninterrupted, open reading frame with two consecutive, 5' Kozak consensus sequences (Kozak, 1995), two Krüppel-related boxes (KRAB) at the N-terminus (Bellefroid *et al.*, 1991; Rosati *et al.*, 1991; Constantinou-Deltas *et al.*, 1992), five zinc finger motifs followed by a potential nuclear localization signal (NLS) at the C-terminus and a 644 bp 3'-untranslated region (Figure 2). Primer extension analysis demonstrated that cDNA clone 6.3 corresponds to an NRIF full-length transcript (data not shown). Depending on whether the first or the second translation initiation codon is used, the predicted NRIF protein consists of 832 or 828 residues and has a mol. wt of 94 080.0 or 93 617.53 Da and a pI of 9.65 or 9.77, respectively.

Mouse NRIF clone 6.3 (Figure 2) is 56% identical overall to the partial human NRIF clone at the amino acid
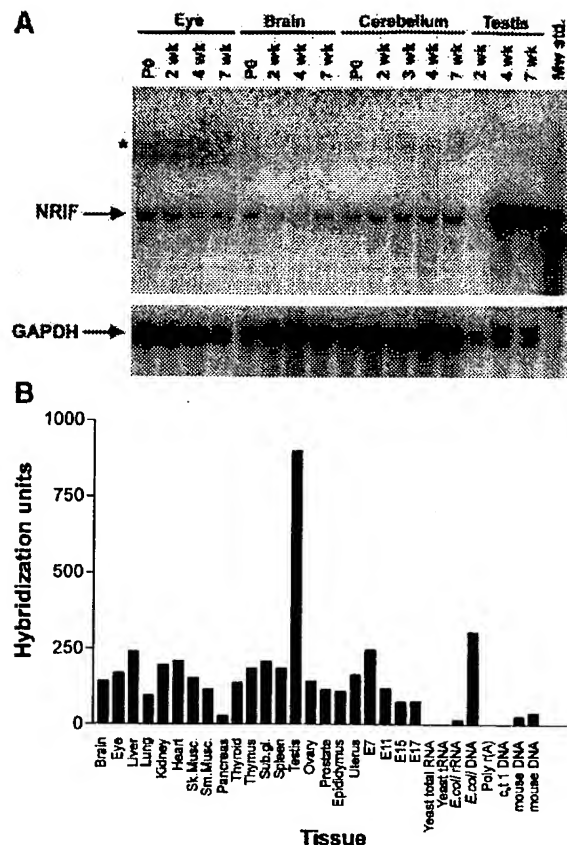
Fig. 3. Tissue distribution of the *nrif* mRNA. Northern blot analysis (A) and poly(A)+ RNA dot blots (B) were used to characterize the RNA banding pattern and accumulation levels of the *nrif* mRNA. (A) The *nrif* cDNA probe detects a double band of ~3.7 kb (arrow) which is most abundant in adult testis, as well as a minor, larger transcript (asterisk). (B) The accumulation levels of the *nrif* mRNA were quantified with a dot blot (Clontech) where the loading was normalized to mRNA expression levels of eight housekeeping genes. The *nrif* mRNA is expressed ubiquitously and at relatively low levels in all tissues examined, except in testis. Sk. Musc, skeletal muscle; Sm. Musc, smooth muscle; Sub. gl., submaxillary gland; E7, E11, E15 and E17, whole embryos at the corresponding embryonic (E) stages. Note that the hybridization signal at E7 is 2-fold higher than at E11 and 3.7-fold higher than at E15. The signal detected in the negative control '*E.coli* DNA' is likely to be due to the probe being prepared from an *E.coli* host.

level. Like human NRIF, it contains five predicted zinc fingers and a pseudo-finger which are 83% identical between human and mouse. The N-terminal half of the protein is much more divergent, sharing only 27% sequence identity. A search in the DNA and protein sequence databases while preparing this manuscript revealed that both the human and the mouse NRIF sequences have been identified independently. Human NRIF corresponds to the partial Zfp2 (DDBJ/EMBL/GenBank accession No. U71598) sequence submitted in 1996 by Petroni *et al.* (unpublished), who identified it as an up-regulated mRNA in an erythroleukaemia cell line. Similarly, a fragment of the mouse NRIF sequence is also present in the expressed sequence tag (EST) database as a 628 bp, Sugano mouse kidney mkia *Mus musculus* cDNA clone (EM_NEW:AI317700, submitted 18.12.98,
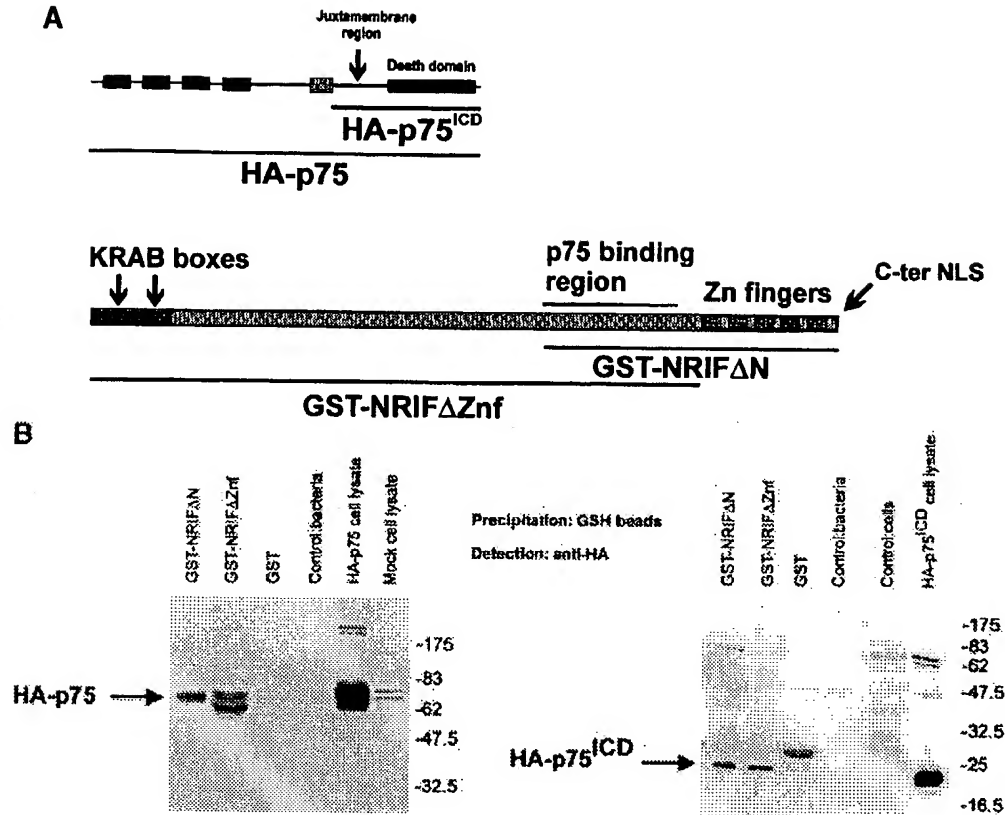
Fig. 4. Biochemical interaction between p75^{NTR} and NRIF. To confirm the interaction observed in yeast, recombinant GST–NRIF fusion proteins (diagrammed in A) were expressed in *E.coli* and immobilized on beads. (B) Whole extracts from 293 cells expressing HA-tagged p75 (left panel) and HA-tagged p75ICD (right panel) were incubated with NRIF-loaded beads in a 'pull-down' assay. Co-precipitated p75^{NTR}, visualized with an anti-HA antibody, was detected only when beads were loaded with GST–NRIF (lanes 1 and 2), but not when the beads contained only GST (lane 3). Negative controls included beads loaded with non-NRIF-expressing bacterial extracts (control bacteria), and cell extracts from mock transfections (control cells). Lanes 5 (left panel) and 6 (right panel) correspond to whole-cell lysates from cells expressing HA-p75 or HA-p75ICD, respectively. The unspecific band detected at ~26 kDa in the right panel is most likely due to an of excess GST protein as seen in Commassie-stained gels (not shown).

WashU-HHMI Mouse EST Project; 99.4% identity in a 170 bp overlap with mouse NRIF).

The chromosomal locations of the mouse and the human NRIF genes have been determined. Mouse NRIF was mapped using a BSS DNA panel and two oligonucleotide primers which detected a single strand conformation polymorphism (SSCP) (Beier *et al.*, 1992; Beier, 1993) between the two parental strains (C57BL/6JEi×SPRET/Ei and SPRET/Ei) in the last intron of the *nrif* gene (see Figure 6A). The locus has been given the Zfp110 symbol and maps to chromosome 7, co-segregating with a large cluster of other loci at the most proximal position (see http://www.jax.org/resources/documents/cmdata/bkmap/maps/7MapPage.html). Given the presence of a zinc finger cluster on this chromosome which is conserved in human chromosome 19 (Stubbs *et al.*, 1996; Shannon and Stubbs, 1998), mapping of the human NRIF gene to the telomeric region of chromosome 19 further confirms that mouse NRIF (corresponding to cDNA clone 6.3) and the human NRIF sequence (originally isolated from the yeast two-hybrid screen) are indeed orthologues.

Mapping the interaction domains

The intracellular domain of p75^{NTR} can be divided into two sub-domains: the juxta-membrane region (residues 244–290) is 95% conserved between chick (Heuer *et al.*, 1990) and human (Johnson *et al.*, 1986; Huang *et al.*, 1996), suggesting some important, conserved function, and the C-terminus (residues 300–399) has homology to other proteins of this receptor family (Feinstein *et al.*, 1995). This latter region can be further subdivided into two segments showing a low degree of homology with the *Drosophila* death protein *Reaper* (residues 300–350 and 366–399). To determine which regions are important for the interaction between NRIF and p75^{NTR}, deletion mutants were tested in the yeast system (Figure 1B). The juxta-membrane sequence was sufficient for interaction with human NRIF and supported growth of the yeast on Leu– plates to a similar extent as the full-length p75ICD. Interestingly, the so-called death domain (Liepinsh *et al.*, 1997) in the remaining C-terminus also interacted with NRIF, suggesting that these two regions may regulate the interaction co-operatively or that NRIF may bind as a dimer to both domains. The first *Reaper*-like region could not be tested alone for interaction because it auto-activated *LEU2* transcription in the absence of NRIF.

To determine which regions of mouse NRIF are responsible for association with p75^{NTR}, deletion constructs of the mouse clone were prepared from an initial construct containing only the last third of the cDNA
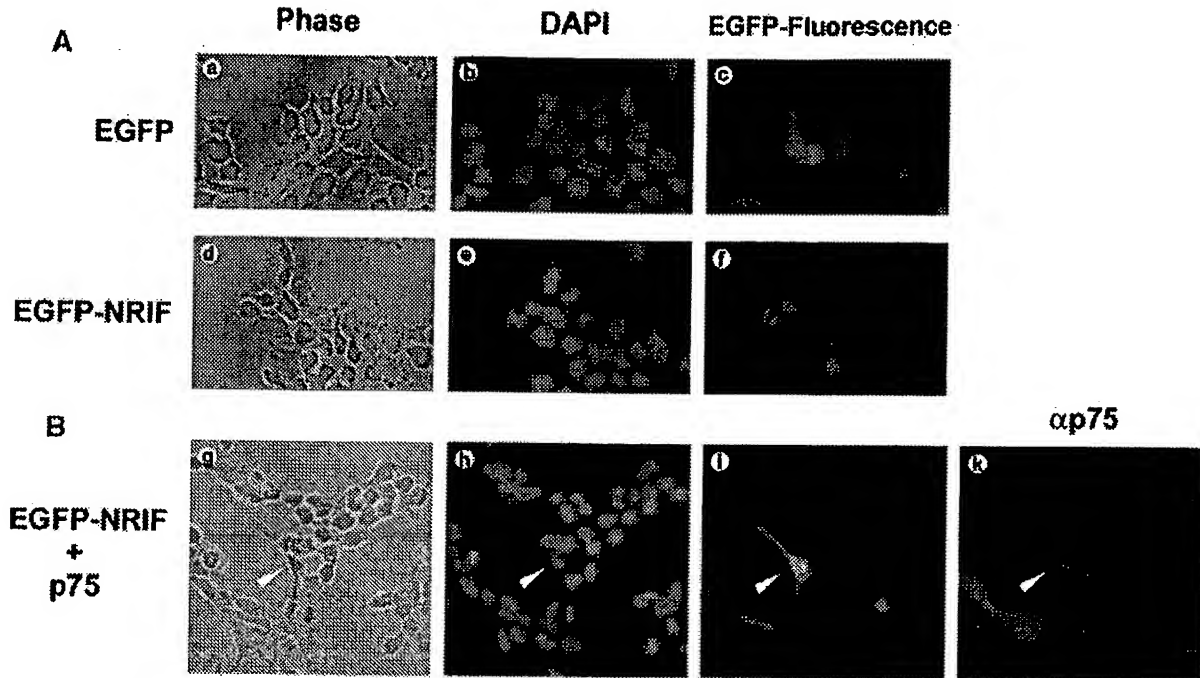
Fig. 5. Subcellular localization of NRIF in transfected 293 cells. (A) 293 cells were transiently transfected with EGFP (a–c) or EGFP–NRIF (d–f). Surviving cells (since EGFP–NRIF expression kills many cells that are removed as floaters) were fixed and stained with DAPI to visualize nuclei (b and e). In contrast to EGFP (c), EGFP–NRIF shows mainly nuclear localization, sometimes with a punctuate appearance (f). (B) 293 cells were transiently co-transfected with EGFP–NRIF and p75^{NTR}, fixed, stained with DAPI (h) and immunostained for p75 (k). Note the difference in efficiency of p75 expression (k) compared with EGFP–NRIF-expressing cells (i). While most cells still show nuclear localization of EGFP–NRIF, co-expression of the p75^{NTR} receptor causes three times as many cells to also express EGFP–NRIF in the cytoplasm (arrowhead), compared with EGFP–NRIF alone.

(region corresponding to the human NRIF fragment originally isolated from the yeast screen). Three mouse NRIF deletion constructs were generated (Figure 1B, right), deleting the pseudo-finger and the zinc fingers, only the zinc finger domain, or the N-terminal region. No significant interaction was observed between p75^{NTR} and the zinc fingers, as would be predicted if this region is a DNA- (or RNA-) binding domain. In contrast, the yeast co-transfected with the NRIF construct lacking the pseudo-finger grew best, suggesting that this remaining region is responsible for the association of NRIF with p75^{NTR}.

NRIF is expressed ubiquitously in mouse tissues

Northern blots probed with the mouse cDNA clone indicated a major double band of ~3.7 kb for the mRNA encoding NRIF, as well as a minor transcript of higher molecular weight, which most likely corresponds to a highly related transcript encoded by a homologous gene (Figure 3A; see Discussion). The NRIF mRNA appears to be ubiquitously expressed albeit at relatively low levels, except in testis (Figure 3A and B). Intact sciatic nerve shows no or very low transcript levels, as indicated by RT–PCR analysis, but NRIF is strongly expressed in dissociated Schwann cells (data not shown). RT–PCR analysis performed in mouse embryonic tissues prepared at E15 indicated that NRIF is also ubiquitously expressed during development, and at higher levels than in the adult. The NRIF mRNA is also expressed in all cell lines analysed (data not shown).

Biochemical interaction of NRIF and p75^{NTR} in vitro

The physical interaction between NRIF and p75^{NTR} was verified biochemically by co-precipitation of the p75 protein expressed in transfected 293 cells with recombinant mouse NRIF protein immobilized on Sepharose beads. Two different portions of the mouse NRIF cDNA were fused to GST: GST–NRIFΔN contains only the last third of the NRIF protein, and GST–NRIFΔZnf expresses full-length NRIF without its zinc fingers (Figure 4A). The levels of recombinant NRIF protein produced and their binding to the Sepharose beads vary depending on the portion of the protein expressed: GST–NRIFΔZnf is induced and bound to the beads at levels comparable to those of the GST moiety alone, while GST–NRIFΔN, containing the five zinc fingers, is poorly expressed, difficult to solubilize under a variety of conditions, and binds inefficiently to the beads. To test the interaction of these two proteins with p75^{NTR}, 293 cells were transfected with rat p75^{NTR} cDNA clones (Figure 4A) expressing either haemagglutinin (HA)-tagged p75^{NTR}ICD (HA-p75ICD) or HA-tagged full p75^{NTR} (HA-p75). As detected by immunoblot, GST–NRIF fusions immobilized on Sepharose beads were capable of binding both the intracellular domain of p75 (HA-p75ICD; Figure 4B right) and the full-length receptor (HA-p75; Figure 4B left) from transfected cells even under high salt conditions, whereas GST alone could not. This confirms the biochemical interaction of NRIF with p75^{NTR}. Curiously, GST–NRIFΔZnf pulls down a slightly different form of p75^{NTR} (Figure 4B, left, lane 2).
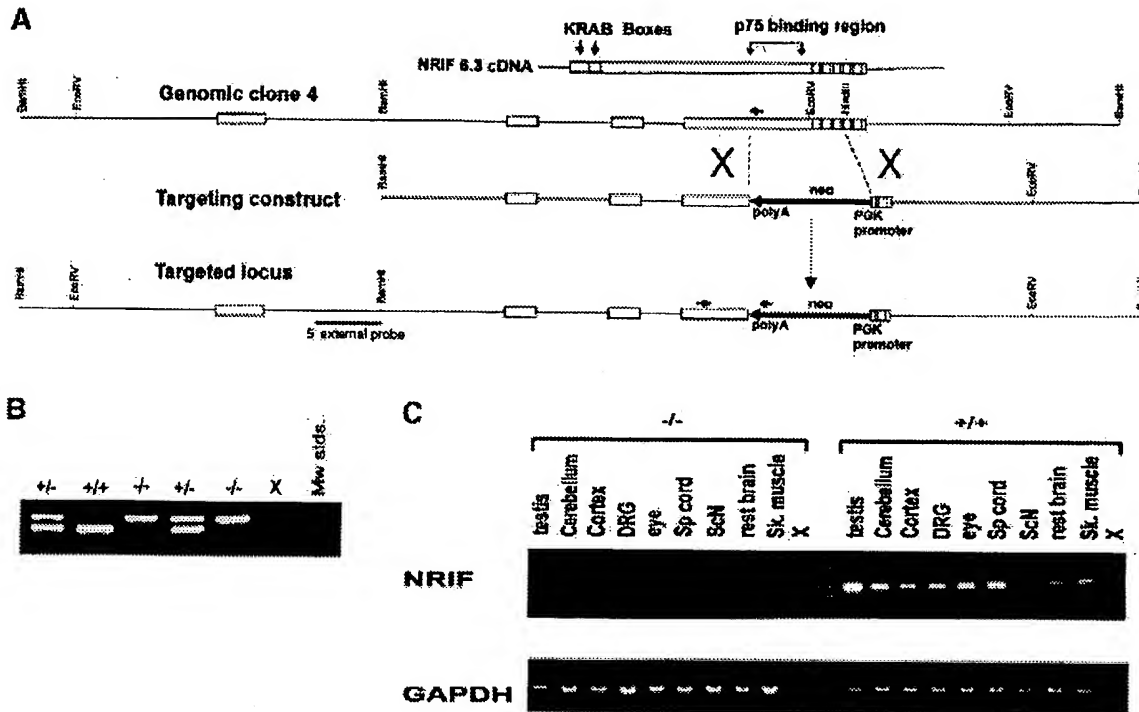
Fig. 6. Genomic targeting of the mouse *nrif* gene. (A) Deletion of the *nrif* exon encoding the p75^NTR-binding region. Shown are the mouse *nrif* cDNA clone 6.3 (predicted amino acid sequence shown in Figure 2), mouse genomic clone 4 isolated from a 129Sv genomic library, and the targeting construct, where the region encoding the p75-interacting portion was replaced by a pGKneo cassette in the antisense orientation. Following homologous recombination in ES cells, the targeted allele can be detected by Southern analysis of genomic *Eco*RV digests probed with the depicted 5' external probe. (B) Genotype analysis. Each PCR (performed on genomic DNA from tail biopsies) contains three primers capable of amplifying the wild-type 521 bp allele (+/+ lanes) and the 572 bp targeted allele (-/- lanes). Heterozygous mice amplify both bands (+/- lanes). Annealing positions for the three primers are indicated by arrows in (A). (C) RT–PCR analysis of *nrif*+/+ and -/- littermates. The absence of transcript from the *nrif* targeted region was confirmed by RT–PCR from RNA prepared from the listed tissues/organs. Wild-type samples contain the *nrif* transcript (upper panel, right), whereas mutant littermates (left) do not express any detectable levels of the targeted region, even after 40 PCR cycles. Equal cDNA levels were used in all PCRs as shown by comparable GAPDH amplification products (lower panel). DRG, dorsal root ganglia; Sp. cord, spinal cord; ScN, sciatic nerve; Sk. muscle, skeletal muscle.

Subcellular localization of the NRIF protein

In order to visualize the subcellular distribution of the NRIF protein in eukaryotic cells, we constructed an enhanced green fluorescent protein (EGFP)–NRIF protein fusion and monitored its expression by fluorescence microscopy. Already at this level, it became clear that over-expression of NRIF can kill transfected cells. Induction of apoptosis was quantified using an enzyme-linked immunosorbent assay (ELISA) method (see Frade and Barde, 1999). Between 20 and 28 h after transfection, NRIF-transfected cells accumulate 86% more free nucleo-somes in their cytoplasm than do mock-transfected cells.

Despite the very low rate of successful transfection and expression, the few viable cells that express EGFP–NRIF show primarily nuclear localization of the protein (Figure 5A). Fluorescence levels are variable, ranging from diffuse to strong, with a granulate pattern which does not co-localize with nucleoli (Figure 5A, panel f, and data not shown). Co-transfection of p75^NTR substantially changes this exclusively nuclear localization: in some co-transfected cells, EGFP–NRIF can be seen both in the nucleus and in the cytoplasm (Figure 5B, panels i and k), or even only in the periphery, co-localizing with p75^NTR. This indicates that the physical interaction between NRIF and p75^NTR can also take place in living cells. Whether the mostly nuclear localization of NRIF is

an artefact of overexpression in transfected cells due to the cytomegalovirus (CMV) promoter is a possibility that cannot be excluded, but expression of NRIF under the control of weaker promoters does not yield detectable levels of expression.

Genomic targeting of the mouse nrif gene

In order to evaluate the functional significance of the interaction between NRIF and p75^NTR, we generated a mouse line with a targeted deletion in the *nrif* gene. The targeting strategy was designed so that the resulting mice would lack the NRIF fragment demonstrated to bind p75^NTR (as mapped in the yeast assay, see Figure 1B). A 6.5 kb *Bam*HI genomic clone (clone 4) isolated from a 129Sv library was identified and selected for genomic targeting of *nrif*. Subcloning of this phage fragment into a pBluescript vector and sequencing revealed the presence of two intronic regions interrupting the spacer region of NRIF (Figure 6A) and confirmed the identity of this clone as the genomic counterpart of the NRIF 6.3 cDNA described above. The 1.2 kb genomic fragment that encodes the p75^NTR-binding domain and four of the five zinc fingers were replaced by a pGK-neo selection cassette, and the resulting targeting construct (Figure 6A) was electroporated into R1 cells. Correct homologous recombination events were identified by Southern analysis

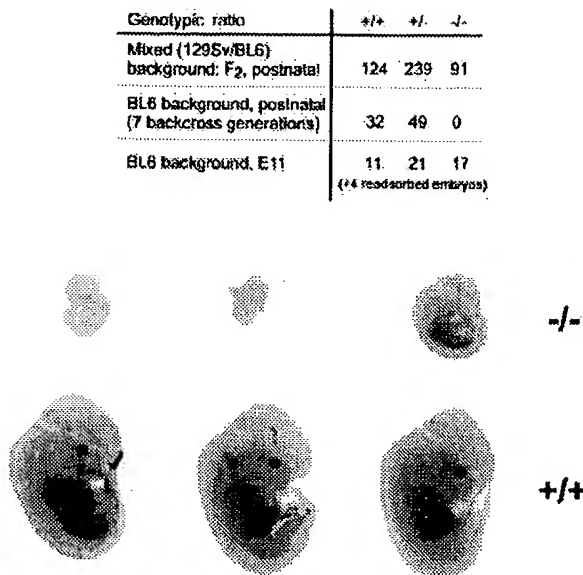| Genotypic ratio	+/+	+/-	-/-
Mixed (129Sv/BL6) background: F₂, postnatal	124	239	91
BL6 background, postnatal (7 backcross generations)	32	49	0
BL6 background, E11	11	21	17 (+4 resorbed embryos)

A

$n(+/+)=4$
$n(-/-)=7$

B

Fig. 7. The *nrif* mutation is viable in mixed (BL6/129Sv) and 129Sv backgrounds, but embryonically lethal in congenic BL6 strains. Table: genotypic ratios obtained for the *nrif* mutation at the F₂ generation (mixed 129Sv/BL6 background) and at the N₇ F₁ generation (seven BL6 backcross generations followed by one intercross), after birth ('postnatal') and at E11. While at the F₂ generation there is only a 20% reduction in the proportion of *nrif⁻/⁻* mice, in a BL6 background the *nrif* mutation is embryonically lethal, and no *nrif⁻/⁻* mice survive beyond E12. Photo: severe growth retardation observed at E12 in *nrif⁻/⁻* embryos in the BL6 background. The photograph shows six members of one litter analysed at E12. Note the difference between the three embryos of the upper row (*nrif⁻/⁻*) as compared with their three wild-type littermates shown below. At E12, *nrif⁻/⁻* embryos are severely growth retarded or in different stages of reabsorption. By E15, all *nrif⁻/⁻* embryos presumably have been resorbed.

using an external 5' probe (Figure 6A). We identified 13 potential candidates amongst 650 isolated G418-resistant clones and, after confirming correct integration with an internal probe, four clones were further selected for injection into BL6 blastocysts. Chimeric mice generated from two of these ES clones (2/4 and 4/39) transmitted the mutation through their germline, and the progeny resulting from the two lines showed the same phenotypic traits at the following generations. A PCR-based assay was developed to show that only the genomic copy corresponding to the 6.3 cDNA was targeted and to screen for the mutant allele (Figure 6B). Complete absence of the targeted region was demonstrated by the lack of RT–PCR product in *nrif⁻/⁻* tissues (Figure 6C).

The effect of the nrif mutation is strongly dependent on the genomic background

At the F₂ generation, *nrif⁻/⁻* mice appear to be viable and fertile, with no obvious gross anatomical defect. Homozygous mutant males appear to be more docile and show an age-dependent, large reduction (up to 70% in young animals) in testis weight, although histological sections through testes show no obvious morphological alterations (data not shown). Genotypic segregation at the F₂ generation slightly deviates from Mendelian: ~20% of the *nrif⁻/⁻* progeny die before or shortly after birth (Figure 7).
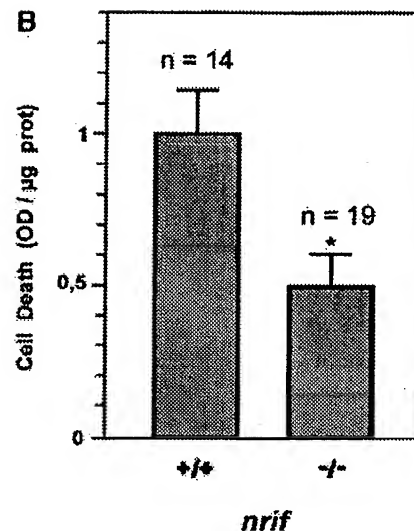
Fig. 8. Reduction of apoptosis in the retina of *nrif⁻/⁻* embryos. Quantification of apoptotic levels in E15.5 retina was done from *nrif⁺/⁺* and ⁻/⁻ littermates. (A) RT–PCR analysis performed on *nrif⁺/⁺* (*n* = 4) and *nrif⁻/⁻* (*n* = 7) retinae demonstrating the presence of p75^NTR and NGF in *nrif⁻/⁻* embryos (B: blank control). (B) Levels of soluble nucleosomes in *nrif⁻/⁻* embryos were normalized to the amount of protein and then to the mean obtained for ⁺/⁺ embryos. In *nrif⁻/⁻*, there is a 50% decrease in the levels of cell death, compared with 48% observed in *p75⁻/⁻* and 56% in *ngf⁻/⁻* embryos (Frade and Barde, 1999).

In order to generate congenic mouse strains harbouring the *nrif* mutation, F₁ heterozygous males were mated to BL6 females and founder F₀ chimeric males were mated to 129Sv females. In each case, this initial mating was followed by nine consecutive backcrosses of heterozygous males to BL6 or to 129Sv females, respectively. Surprisingly, to date, interbreeding of heterozygous animals at the N₆(BL6) backcross generation could never produce viable *nrif⁻/⁻* progeny (Figure 7), whereas the 129Sv lineage produces *nrif⁻/⁻* pups even after 10 generations of consecutive backcrosses. Embryonic death in the BL6 background seems to occur around E12, since no living *nrif⁻/⁻* embryos were found at E13, E14 or E15, whereas several litters examined at E11 contained living homozygous mutants in Mendelian proportions (Figure 7). With wide variability, E12 *nrif⁻/⁻* embryos showed very

different degrees of growth retardation in comparison with their wild-type littermates (Figure 7). Such a lethal phenotype could be rescued easily by interbreeding of heterozygous mice in the two congenic strains [$nrif^{+/-}$ N$_{10}$(BL6)×$nrif^{+/-}$N$_{10}$(Sv129)], indicating that only the BL6 context cannot support the $nrif$ mutation in homozygosis.

NRIF plays a role in developmental cell death in the mouse retina

In order to evaluate the possible involvement of NRIF in the pro-apoptotic pathway of p75^{NTR} *in vivo*, we quantified cell death levels in the retina of wild-type and $nrif^{-/-}$ E15.5 F$_2$ littermates. Nine litters with both $^{+/+}$ and $^{-/-}$ embryos were analysed, and the amounts of free nucleosomes in retinal extracts quantified as described before (Frade and Barde, 1999). Despite variability, as previously observed with both the ngf and the *p75^{NTR}* mutations (see Frade and Barde, 1999), we observed a significant reduction in the levels of cell death in the $nrif^{-/-}$ group when compared with their wild-type littermates (Figure 8). This reduction is quantitatively indistinguishable from what has been observed in $p75^{-/-}$ and $ngf^{-/-}$ mutants (Frade and Barde, 1999).

Discussion

Accumulating evidence suggests that p75^{NTR} can initiate programmed cell death in a number of specific cell types (Casaccia-Bonnefil *et al.*, 1998). However, unlike the well-characterized signal transduction pathways used by the trk receptors (Kaplan and Miller, 1997), the signalling molecules recruited by p75^{NTR} in its pro-apoptotic function are still unknown. To date, only TRAF6 has been reported as a p75^{NTR}-interacting molecule mediating p75^{NTR} effects, and it seems to be involved in the activation of NF-κB (Khursigara *et al.*, 1999). To gain more insight into the underlying mechanisms of p75^{NTR} signalling, we used the yeast two-hybrid system and identified NRIF. This novel protein associates with the cytoplasmic domain of p75^{NTR}. Together with TRAF6, NRIF represents the first adaptor molecule found to interact directly with p75^{NTR} to transduce signals elicited by neurotrophin binding at the cell membrane.

The mouse $nrif$ gene encodes a 94 kDa zinc finger protein of the Krüppel family. Although originally isolated on the basis of its interaction with the intracellular domain of p75^{NTR} in yeast cells (Figure 1), our pull-down assays demonstrated that full-length p75^{NTR} from mammalian cells also interacts with NRIF (Figure 4B, left). Further, the ICD of p75^{NTR} alone was precipitated by NRIF (Figure 4B, right), suggesting that the extracellular portion of the receptor is not required for the interaction. As mapped in yeast cells, NRIF and p75^{NTR} appear to interact between the region immediately N-terminal to the zinc fingers of NRIF, and two discrete portions of the p75ICD (Figure 1): the highly conserved juxta-membrane sequence of p75^{NTR}, shown to be the TRAF6-binding domain (Khursigara *et al.*, 1999), and the death domain (Liepinsh *et al.*, 1997). Deletion of either region of p75ICD did not prevent interaction with NRIF (Figure 1), indicating that NRIF can bind to both regions, possibly as a dimer. In yeast cells, the zinc fingers alone do not participate in the

binding of NRIF to p75^{NTR} (Figure 1), indicating that they are potentially free and capable of binding DNA or RNA.

The primary structure of NRIF (Figure 2), including its five zinc fingers, two N-terminal KRAB domains and a putative NLS, suggest that it may translocate to the nucleus and regulate gene expression. Indeed, several zinc finger proteins containing KRAB domains have been shown to be transcriptional repressors (Witzgall *et al.*, 1994). The subcellular distribution of NRIF supports such a role also for this interactor: when expressed in 293 cells, EGFP–NRIF is primarily nuclear, but upon co-expression of p75^{NTR} both proteins can be seen co-localizing outside the nucleus (Figure 5B). This raises the possibility that the p75^{NTR}–NRIF signalling system may function in a manner similar to other receptor–transcription factor associations, such as Notch and Su(H) (Weinmaster, 1997) or the transforming growth factor-β (TGF-β) receptors and SMADs (Heldin *et al.*, 1997). Upon activation of these receptors, their associated factors are released and translocate to the nucleus where they mediate their respective functions.

The $nrif$ mRNA is expressed ubiquitously, but at higher and constant levels in the embryo, while in the adult mouse accumulation levels of the $nrif$ mRNA vary among tissues and organs (Figure 3). The presence of a human $nrif$ homologous and syntenic gene in the human genome further supports an evolutionarily conserved, important function. Furthermore, both the mouse and the human genome contain a second, highly related $nrif$ gene which most likely arose as a result of a gene duplication. Zinc finger gene clusters in mouse chromosome 7 and human chromosome 19 are currently being fully sequenced (Lawrence Livermore Laboratories), so it will be interesting to see whether more members of the $nrif$ family are described in the future.

In support of an essential role for NRIF, all cell lines examined express the $nrif$ mRNA, as determined by RT–PCR studies (data not shown). However, the cellular function of NRIF has proven difficult to assess due to the very low levels of detectable expression upon transfection of the cDNA in a variety of cell lines, e.g. COS7, BHK, CV1, NIH-3T3 and HN10. Even 293 cells, known and widely used for their high efficiency of transfection, only rarely express transfected $nrif$, indicating that there may be a very narrow range of accumulation of the NRIF protein still allowing cell viability and/or proliferation. Overexpression of the NRIF protein seems to be toxic for transfected cells, and the proportion of successfully transfected cells that can express tagged $nrif$ cDNAs is very low. It is possible that only cells in a particular stage of their cell cycle allow overexpression of NRIF.

To evaluate more directly the physiological significance of the NRIF–p75^{NTR} interaction *in vivo*, we generated $nrif^{-/-}$ mice by homologous recombination. Based on our mapping studies in yeast (Figure 1) and with recombinant NRIF protein (Figure 4), we replaced the p75^{NTR}-binding region of NRIF with a pGKneo selection cassette (Figure 6). The resulting $nrif^{-/-}$ mice express a truncated NRIF transcript lacking the p75^{NTR}-binding region and four of its five zinc fingers. These mice show a reduction in cell death in embryonic retina (Figure 8) which is virtually identical in its extent to what is observed in the $p75$ and ngf mutants (Frade and Barde,

1999), pointing to these three genes being operative in the same pathway. Our results are compatible with the hypothesis that during the period of naturally occurring cell death in the developing retina, NRIF actively participates in the apoptotic process mediated by p75^{NTR}. Several zinc finger proteins of the C2H2 type have already been shown to participate in programmed cell death. For example, TIEG, a TGF-β-inducible gene in pancreatic epithelial cells, has been shown to induce apoptosis when overexpressed (Cook et al., 1998). Similarly, Zac1 is a tumour suppressor gene expressed only in brain and pituitary gland and, like p53, it can regulate apoptosis and cell cycle arrest (Spengler et al., 1997). Krox-20, originally identified as a serum response immediate early gene (IEG) (Chavrier et al., 1988), activates transcription from a specific DNA recognition element (Swirnoff and Milbrandt, 1995), controls segmentation during hindbrain development (Schneider-Maunoury et al., 1993) but also regulates susceptibility to apoptosis in developing Schwann cells (Zorick et al., 1999). Like Krox-20 and Nurr77 (Watson and Milbrandt, 1990), nrif is also induced in cultured cells by NGF; yet a direct interaction of a KRAB-containing zinc finger protein with a neurotrophin receptor has so far only been shown for NRIF. Presumably, binding of NGF to p75^{NTR} induces a conformational change in the receptor which then releases NRIF. NRIF, in turn, could translocate to the cell nucleus where its zinc fingers would bind specific DNA sequences, while its KRAB domains could silence basal and inducible transcription. Future work will attempt to identify downstream targets of the NRIF protein, the p75^{NTR} gene itself being a candidate. Indeed, p75^{NTR} mRNA levels are up-regulated in nrif$^{-/-}$ mice (E.Casademunt, unpublished data, and Figure 8).

Unexpectedly, the phenotype of the nrif mutation appears to be masked in the mixed BL6/Sv129 background. Although at the F$_2$ generation (BL6:Sv129 50:50 hybrids) the mutation causes only limited lethality, after seven backcross generations to the BL6 strain nrif$^{-/-}$ mice are no longer viable beyond E12 (Figure 7). The fact that the lethal phenotype can be rescued by only one generation of mating to nrif$^{+/-}$ mice of Sv129 background strongly indicates that the BL6 genome cannot support the homozygous mutation. This situation is in principle analogous, but exactly opposite in the details to that described for the Pax-2 (Schwarz et al., 1997), Egfr (Threadgill et al., 1995) and Tgfβ1 mutations (Bonyadi et al., 1997), all of which have lethal consequences in a congenic Sv129 background. Our results are a further illustration of the need to evaluate the effect of targeted mutations in different congenic backgrounds (see e.g. Banbury Conference on Genetic Background in Mice, 1997; Frankel, 1998).

In conclusion, NRIF is the first zinc finger protein described to interact with the p75^{NTR} receptor and to play an active role in developmental programmed cell death. NRIF may therefore represent a new class of receptor-associated transcription factors important for regulating cell viability.

Materials and methods

Interaction trap
Plasmid vectors and yeast strain EGY48 required for the interaction trap were all generously provided by R.Brent (Gyuris et al., 1993). The bait p75ICD construct was generated by PCR amplification from a full-length rat p75^{NTR} cDNA clone (provided by E.Shooter) using the following primers: 5'-ATC GAA TTC TTC AAG AGG TGG AAC AG and 5'-GAG GGA TCC GTG AGT TCA CAC TGG GG, and in-frame cloning into the EcoRI–BamHI sites of the yeast bait lexA fusion pEG202 vector. The interaction trap was performed as previously described (Gyuris et al., 1993), using a human, 22-week-old, fetal, frontal cortex cDNA library cloned into the pJG4-5 plasmid (provided by D.Krainc). Out of 1.3 × 10^6 colonies plated, 200 grew under selective conditions and 17 of them showed galactose-dependent growth (conditions for induction of the library). Yeast DNA was isolated and retested for specific interaction with the p75^{NTR}ICD bait and with additional baits, e.g. bicoid and GST. Deletion constructs of rat p75^{NTR} were generated by PCR amplification of the cDNA encoding residues 244–290 and 295–396, and subcloned into pEG202. Deletion constructs of mouse nrif were PCR amplified from the cDNA encoding residues 521–661, 521–689 and 690–832, and subcloned into pJG4-5.

Cloning of the mouse nrif cDNA and genomic clones
Approximately 7.5 × 10^5 p.f.u. of a P1 mouse brain cDNA library in λZAPII (Stratagene) were plated and transferred to nylon membranes (Hybond N, Amersham). The filters were screened with a 0.9 kb, EcoRI–XhoI human nrif cDNA probe including the zinc finger domain, radiolabelled by random priming (Amersham). Hybridization was carried out with 5× SSC, 5× Denhardt's, 50 mM Tris–HCl pH 7.4, 1% SDS, 40% formamide and 100 μg/ml salmon sperm DNA at 42°C overnight. The filters were washed at 65°C in 1× SSC, 0.1% SDS. Positive clones were in vivo excised using Exassist phage and SOLR recipient cells (Stratagene), and recovered as pBluescript SK plasmids. Sequence analyses were done with the MacMolly or the Wisconsin Genetics Computer Group software.

Approximately 2 × 10^6 genomic clones from a mouse (129/Sv) genomic library made in the λFIXII vector (Stratagene) and plated in LE392 host cells were screened using two radioactively labelled, overlapping fragments of the mouse NRIF 6.3 cDNA (NcoI–EcoRI, 1582 bp and NcoI–BamHI, 653 bp). Hybridization and washing were done according to Church and Gilbert (1984). Six out of 14 independent clones which hybridized with both probes appeared to be independent isolates of the same gene, and all further work was with genomic clone 4 (Figure 6A).

Northern and RT–PCR analysis
Total RNA was extracted following a modified RNAzol B method (P.Chomczynski, Wak-Chemie Medical GmbH) from mouse embryonic (E15.5) and adult (1–2 months old) tissues. For Northern analysis, 20 μg of total RNA was glyoxylated, electrophoresed on 1.2% agarose gels in 10 mM NaH$_2$PO$_4$/Na$_2$HPO$_4$ pH 6.5 and vacuum-transferred to Hybond N membranes. nrif mRNA levels were quantified using an RNA Master Blot (Clontech) following the manufacturer's instructions and a Molecular Dynamics phosphorimager. Northern blots were probed with a 1.1 kb SpeI–EcoRV fragment of the nrif cDNA which excludes the zinc finger region. RT–PCRs were done from 500 ng of total RNA essentially as described (Friedel et al., 1997), using the following PCR primers: 5'-GTG ATG CTA GAG ACC TTG GG; 5'-CAC CAT GGC ATC GCT ACT GTC C. Because of the low levels of expression of the nrif mRNA, the PCR requires 5-fold higher levels of starting cDNA than those required to amplify the GAPDH transcript. Cycle parameters were 94°C 4 min; 94°C 30 s, 58°C 30 s, 72°C 40 s, 35 cycles.

Recombinant NRIF protein expression
Production of recombinant NRIF proteins in E.coli JM109 was induced for 4 h at room temperature by 0.5 mM isopropyl-β-D-thiogalactopyrano-side (IPTG) with 0.1 mM ZnCl$_2$. Bacterial pellets were resuspended in lysis buffer [50 mM HEPES pH 7.9, 1% Triton X-100, 5 mM dithiothreitol (DTT), 0.5 M NaCl, 0.1 mM ZnCl$_2$, 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 μg/ml aprotinin, 5 μg/ml leupeptin] and lysed by sonification. Soluble protein obtained by centrifugation was bound to glutathione–Sepharose 4B beads (Pharmacia Biotech) for 4 h at 4°C. Control beads were prepared by using the same host bacteria lacking any plasmid. Bound beads were washed extensively with 1× phosphate-buffered saline (PBS), 0.3 M NaCl, 0.1 mM ZnCl$_2$. Protein binding to the beads was assessed by SDS–PAGE analysis of a small aliquot eluted with Laemmli sample buffer.

Cell culture and transfection
293 cells (Graham et al., 1977) were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco-BRL) supplemented with 10% fetal

calf serum (Sigma) and 1% penicillin/streptomycin (Gibco-BRL) at 37°C and 5% CO_2. Transient transfection was by the calcium phosphate precipitation method according to Chen and Okayama (1987), seeding 2.5×10^6 cells onto 10 cm plates the day preceding transfection. Transfection efficiency was estimated by parallel transfection with a pCMV-GFP vector (pEGFP-N1, Clontech).

'Pull-down assays'/Western blot
Two days after transfection, cells were washed with ice-cold PBS and lysed in 1 ml of lysis buffer/plate [25 mM HEPES pH 7.5, 150 mM NaCl, 10% glycerol, 0.8 % NP-40, 4 mM Pefabloc (Boehringer Mannheim), 5 µg/ml aprotinin, 5 µg/ml leupeptin] followed by centrifugation at 15 000 g, 4°C for 20 min. The supernatant (300 µl) was mixed with binding buffer (900 µl; 25 mM HEPES pH 7.5, 150 mM KCl, 5 mM $MgCl_2$, 0.13 mM $ZnCl_2$, 5 mM DTT, 4 mM Pefabloc, 5 µg/ml aprotinin, 5 µg/ml leupeptin), and incubated overnight with a 50 µl bed volume of protein-loaded glutathione–Sepharose 4B beads (see above). After washing the beads once with washing buffer 1 (WB1: 40 mM HEPES pH 7.5, 300 mM NaCl, 5 mM $MgCl_2$, 0.1 mM $ZnCl_2$, 1 mM DTT) and twice with WB2 (150 mM NaCl, otherwise like WB1), the proteins were eluted by boiling with 50 µl of 2× Laemmli buffer for 8 min, separated on a 10 or 12% SDS–polyacrylamide gel and transferred to PVDF membranes (Immobilon-P, Millipore). Protein blots were blocked with 5% non-fat milk powder in TBST (25 mM Tris pH 7.5, 150 mM NaCl, 0.1% Tween-20), incubated overnight at 4°C with 0.5 µg/ml anti-HA monoclonal antibody (Boehringer Mannheim) in blocking buffer, washed five times with TBST, incubated with goat anti-mouse IgG-POD (Pierce, 1:10 000 in blocking buffer) for 1 h at room temperature, washed again and finally developed using the ECL detection system (Amersham).

Construction of p75^{NTR} and NRIF expression plasmids
The intracellular domain of p75^{NTR} (starting with Lys251) used for pull-down experiments was amplified by PCR from rat p75^{NTR} cloned into pcDNA3 (Invitrogen) with the following primers: 5'-GTC AAG CTT GCC ACC ATG TAT CCT TAT GAC GTG CCT GAC TAT GCC GGG GGA AAG AGG TGG AAC AGC TG (which introduces a Kozak consensus sequence followed by a HA tag and a glycine linker), and 5'-GTC GCG GCC GCT CAC ACT GGG GAT GTG GCA G (primer 'p75end'), and then subcloned into HindIII–NotI-digested pRc/CMV (Invitrogen), creating HA-p75ICD. Full-length -p75 was amplified by PCR from the same template with primers 5'-GTC GCT AGC TTA TCC TTA TGA CGT GCC TGA CTA TGC CGG GGG AAA GGA GAC ATG TTC CAC AGC and 'p75end' and subcloned into pRc/CMV AC7 (Bibel et al., 1999).
The full-length NRIF coding sequence (residues 2–832) was amplified by PCR with primers 5'-GAGGACAGTATGGCTTCC and 5'-CTAC-AATTTGGGCTGCTTC, and subcloned into pEGFP-C1, fusing EGFP at the N-terminus of NRIF to produce pEGFP-NRIF, or into pFLAG-CMV-2 creating N-terminally flagged NRIF (pFLAG-NRIF). The GST–NRIFΔZnf construct was made by subcloning the NRIF cDNA sequence excluding the zinc fingers from pFLAG-NRIF into pGEX-2T (Pharmacia). GST–NRIFΔN was generated by PCR amplification from cDNA clone 6.3 (Figure 6A) and subcloning into pGEX-4T-1 (Pharmacia).

Immunocytochemistry
Transiently transfected 293 cells grown on 13 mm glass coverslips (coated with poly-L-lysine, Sigma, 0.01%) were fixed with 2–4% paraformaldehyde in PBS for 15 min at room temperature, permeabilized with 0.2% Triton X-100/PBS, blocked in 10% bovine serum albumin, 10% normal goat serum (NGS)/PBS for at least 1 h at room temperature, and incubated overnight at 4°C with a polyclonal antibody against the intracellular domain of p75^{NTR} in PBS/5% NGS. After three 10 min washes with PBS, cells were incubated for 1 h at room temperature with LRSC-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories) and 4',6-diamidino-2-phenylindole (DAPI) at 0.5 µg/ml in PBS/5% NGS. Following five 10 min washes with PBS, cells were mounted in 'Immuno Fluore Mounting Medium' (ICN Biochemicals) and analysed using fluorescence microscopy.

Nrif gene targeting. Breeding scheme
To target the nrif gene, the genomic region corresponding to the region between residues 520 and 777 (Figures 2 and 6A) was replaced by the neo cassette from the pGKneopA vector (from Soriano et al., 1991), resulting in a targeting construct where the 5' arm was 3.2 kb, the 3' arm was 2.3 kb and the transcription orientation of the neo cassette was

opposite to that of the nrif gene. R1 ES cells (Nagy et al., 1993) were electroporated, selected with G418 and screened by Southern blot with an external 5' probe (Figure 6A) which recognizes a 6.5 kb wild-type band and a 9 kb mutant band in a genomic EcoRV digest.
BL6 mice were of the C57BL/6NJCrl (Charles River Laboratories) strain; Sv129 mice of the 129SvPas substrain. Chimeric mice were generated by transfer of BL6 blastocysts injected with targeted ES cells into pseudopregnant females. After generation of F_2 viable homozygous offspring, an intensive programme of backcrossing to BL6 and 129Sv was started by mating F_1 heterozygous females to BL6 males and founding chimeric mice to 129Sv females. In each case, heterozygous females were mated to BL6 and 129Sv males, respectively, for 10 consecutive generations in order to obtain congenic strains of each background. Mice were genotyped by Southern and PCR analysis from tail or yolk sac DNA extracted as described before (Laird et al., 1991; Hogan et al., 1994). PCR primers for nrif genotyping were as follows: 5'-TCC ATG ACA CGA GGT TTA CG, 5'-CAG AAG TGC TAA GTT CCT CG and 5'-GAT TCG CAG CGC ATC GCC TTC, and cycle parameters: 94°C 4 min; 94°C 30 s, 57°C 30 s, 72°C 1 min, 35 cycles. The two first primers amplify the 521 bp wild-type band, whereas the first and third primer amplify the 572 bp targeted band.

Quantification of cell death in E15.5 retina
Heterozygous F_1 mice were mated until a vaginal plug (E0.5) was observed. Fifteen days later (E15.5), embryonic retinas were prepared and processed as described in Frade and Barde (1999), with duplicate measurements from each retina. The results are based on the analysis of nine litters (78 embryos). Six litters generated by homozygous +/+ and –/– parents were also analysed to evaluate the degree of variability between littermates of the same litter.

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Strain-dependent embryonic lethality in mice lacking the retinoblastoma-related p130 gene

Jennifer E. LeCouter, Boris Kablar, Peter F. M. Whyte, Chuyan Ying and Michael A. Rudnicki*

Institute for Molecular Biology and Biotechnology, McMaster University, 1280 Main Street West, Hamilton, Ontario, Canada L8S 4K1
*Author for correspondence (e-mail: rudnicki@mcmaster.ca)

SUMMARY

The retinoblastoma-related p130 protein is a member of a conserved family, consisting of Rb, p107 and p130, which are believed to play important roles in cell-cycle control and cellular differentiation. We have generated a null mutation in *p130* by gene targeting and crossed the null allele into Balb/cJ and C57BL/6J strains of mice. In an enriched Balb/cJ genetic background, *p130*$^{-/-}$ embryos displayed arrested growth and died between embryonic days 11 and 13. Histological analysis revealed varying degrees of disorganization in neural and dermamyotomal structures. Immunohistochemistry with antibody reactive with Islet-1 indicated markedly reduced numbers of neurons in the spinal cord and dorsal root ganglia. Immunohistochemistry with antibody reactive with desmin indicated a similar reduction in the number of differentiated myocytes in the myotome. The myocardium of mutant embryos was abnormally thin and resembled an earlier staged two-chambered heart consisting of the bulbus cordis and the ventricular chamber. TUNEL analysis indicated the presence of extensive apoptosis in various tissues including the neural tube, the brain, the dermomyotome, but not the heart. Immunohistochemistry with antibody reactive with PCNA revealed increased cellular proliferation in the neural tube and the brain, and decreased proliferation in the heart. The placentas of *p130*$^{-/-}$ embryos did not display elevated apoptosis and were indistinguishable from wild type suggesting that the phenotype was not due to placental failure. Following a single cross with the C57BL/6 mice, *p130*$^{-/-}$ animals were derived that were viable and fertile. These results indicate that *p130* in a Balb/cJ genetic background plays an essential role that is required for normal development. Moreover, our experiments establish that second-site modifier genes exist that have an epistatic relationship with *p130*.

Key words: p130, Balb/C, Embryonic lethality, Mouse, Retinoblastoma

INTRODUCTION

In the developing embryo, combinatorial signals elicit appropriate patterning and morphogenesis via regulatory networks that control stem-cell determination, proliferation, differentiation and programmed death (Slack, 1992). The retinoblastoma (Rb) family, including *Rb, p107* and *p130*, plays an integral role in these regulatory pathways, in part by negatively regulating E2F-dependent transcription (Weinberg, 1995). Moreover, an important role for p130 in repressing cell cycle progression during differentiation is supported by the observation that p130:E2F complexes are predominant in differentiated cells (Muller, 1995; Nevins et al., 1997). Different members of the Rb-family also regulate the activity of other transcription factors including the developmentally important paired homeodomain-containing proteins MHox, Chx10 and Pax-3 (Wiggan et al., 1997), as well as the transcriptional control proteins C/EBP and c-Myc (Chen et al., 1996; Weinberg, 1995; Whyte, 1995).

Rb-family members differentially bind cyclins and their associated kinases (cyclin-dependent kinases or Cdks) resulting in the differential phosphorylation of Rb-members

during progression through the cell cycle. Consequently, Rb, p107 and p130 are hypophosphorylated during different phases of the cell cycle allowing the formation of complexes that contain different E2F transcription factors (Nevins et al., 1997). The multimember E2F-family of transcription factors regulates the transcription of many genes, for example, thymidylate synthase, ribonucleotide reductase M2, DHFR, B-myb and cdc2 that are involved in DNA synthesis and cell-cycle progression. Complexes containing hypophosphorylated Rb-family members are believed to bind promoters at E2F sites and inhibit transcription by recruiting HDAC1, a histone deacetylase, to repress gene expression via chromatin remodeling (Brehm et al., 1998; Luo et al., 1998; Magnaghi-Jaulin et al., 1998). Presumably, the cyclic activation and repression of E2F-regulated genes facilitates appropriate gene expression and hence progression through the cell cycle (Muller, 1995).

Mice carrying targeted mutations in *Rb* display phenotypes supportive of a role for Rb in cellular differentiation. Homozygous mutant embryos die in utero between day 13.5 and day 15.5 of gestation and exhibit defects in erythropoiesis and extensive cell death in the central nervous system (Clarke

et al., 1992; Jacks et al., 1992; Lee et al., 1992, 1994; Macleod et al., 1996). Chimeras containing both wild-type and Rb-deficient cells are viable, but exhibit adrenal medulla hyperplasias, pituitary tumors and lens cataracts (Maandag et al., 1994; Williams et al., 1994). Unlike Rb-deficient embryos, Rb⁻ᐟ⁻:wild-type chimeras contain mature Rb-deficient erythrocytes suggesting that erythroid differentiation is delayed rather than blocked in the absence of Rb.

Mice lacking either *p107* or *p130* in a mixed 129/Sv:C57BL/6J genetic background exhibit no overt phenotype, are viable and fertile, and embryonic fibroblasts derived from the mutants display normal cell-cycle kinetics (Cobrinik et al., 1996; Herrera et al., 1996; Hurford et al., 1997; Lee et al., 1996). Embryos lacking both *Rb* and *p107* die in utero 2 days earlier than *Rb*-deficient embryos and exhibit apoptosis in the liver and central nervous system suggesting some redundancy in function. Compound mutant mice lacking both *p130* and *p107* die soon after birth and exhibit defective endochondral bone development likely due to a deficiency in osteoblast differentiation. Taken together, these data suggested that p107 and p130 have relatively subtle roles in regulating the cell cycle and that a significant degree of overlap in function exists between the proteins (Cobrinik et al., 1996; Lee et al., 1996).

We have independently derived a targeted null mutation in *p130* into the germline of mice. In our experiments, we bred chimeras with mice from the Balb/cJ strain. Surprisingly, we observed that mice lacking *p130* displayed an embryonic lethal phenotype associated with reduced cellular differentiation and increased apoptosis. These data strongly support the assertion that *p130* in a Balb/cJ genetic background plays an essential role that is required for normal development. Moreover, the observed strain-dependence of the phenotype suggests that second-site modifier genes exist that have an epistatic relationship with *p130*.

MATERIALS AND METHODS

Generation of p130 mutant mice

A 13 kb fragment of the *p130* locus was cloned from a J1 genomic library and was used to construct a targeting vector containing 3 kb of 5′- and 7.2 kb of 3′-homologous sequence. The PGK-neo expression cassette was inserted in the opposite transcriptional orientation to p130 immediately downstream of the codon encoding aa 106 (Fig. 1). The *p130* targeting vector was linearized with *Not*I and gene targeting performed with the J1 line of ES cells as described previously (Rudnicki et al., 1992). Targeting events were detected by Southern analysis of *Eco*RV-digested genomic DNA using a 5′ flanking probe. Two independent targeted lines were injected into Balb/cJ blastocyst stage embryos to generate chimeras. Chimeras were subsequently mated to Balb/cJ females and the resulting heterozygous mice were bred to produce homozygous mutant mice. Care of animals was in accordance with institutional guidelines.

Northern and immunoblot analysis

Northern analysis of total RNA (10 µg) was performed using standard techniques with the full-length mouse *p130* cDNA as probe (LeCouter et al., 1996). Immunoblot analysis was performed as follows. Protein lysates were prepared by lysing in modified TNE (1 mM NaV and 10 µg/ml PMSF, aprotinin, pepstatin and leupeptin) or EBC lysis buffer (50 mM Tris HCl, pH 7.5, 0.5% NP40, 150 mM NaCl and protease inhibitors). Protein (30 µg) was electrophoresed on 7.5% SDS-

polyacrylamide gels and transferred to PVDF membranes. Membranes blocked with 5% skim milk powder in TBST were incubated for 1 hour at room temperature with anti-p130 antibody C-20 (Santa Cruz) diluted 1:500. Following five washes in TBST, secondary antibody (diluted 1:2000) was incubated at RT for 1 hour. After five TBST washes, proteins were visualized by ECL detection (Amersham).

Histopathology and immunohistochemistry

Preparation, fixation, sectioning and staining of tissue samples for light microscopy of histological preparations were performed as described previously (Kablar et al., 1997). Following timed matings, embryos were isolated at different stages of gestation and fixed in 4% paraformaldehyde in PBS for 12-20 hours. Processing and staining with antibodies reactive with Isl-1 (Developmental Hybridoma Bank), desmin (Dako) and PCNA (Dakopatts) was performed as previously described (Kablar et al., 1997). TUNEL analysis was performed with the ApopTag Kit (Oncor). All sections were lightly counterstained with hematoxylin.

RESULTS

Targeted inactivation of *p130* in mice

The *p130* gene was disrupted by homologous recombination in J1 embryonic stem (ES) cells using standard techniques (Rudnicki et al., 1992). The *p130* targeting vector was constructed by inserting the PGK-neo cassette (McBurney et al., 1991) into a 1 kb deletion originating from a *Bam*HI site introduced into an exon immediately downstream of the codon encoding aa 160 to a *Kpn*I site within the downstream intron. The PGK-neo cassette was inserted in the opposite transcriptional orientation to *p130* (Fig. 1A). Approximately 1% of G418-resistant clones contained the targeted *p130* allele as revealed by Southern analysis. A probe upstream of the targeting vector detected an 18.5 kb *Eco*RV fragment from the wild-type *p130* allele, whereas an 7.5 kb *Eco*RV fragment was detected following homologous recombination (Fig. 1B).

Chimeras were generated following microinjection of two independently derived targeted ES lines into Balb/cJ blastocysts. Southern analysis of tail DNA in *p130*⁺ᐟ⁻ germline progeny revealed the predicted restriction fragment length polymorphism (not shown). Two independent *p130* mutant mouse lines were derived into the germline and the observed homozygous embryonic lethal phenotype was completely identical in all experiments and are hereafter discussed together (see below).

To confirm that the engineered disruption of *p130* by PGK-neo had generated a null mutation, we examined expression of p130 at the level of mRNA and protein by northern and immunoblot analysis. RNA and protein was isolated from E14 embryos from an enriched C57BL/6 genetic background as previously described (LeCouter et al., 1998). Northern analysis was performed on total RNA using the full-length mouse *p130* cDNA as probe (Fig. 1C). The mature 4.9 kb *p130* mRNA was readily detected in wild-type total RNA (Fig. 1C, lane 1) and the level of *p130* mRNA was reduced by about half in *p130*⁺ᐟ⁻ total RNA (Fig. 1C, lane 2). However, no *p130* mRNA was detected in RNA isolated from *p130*⁻ᐟ⁻ samples (Fig. 1C, lane 3).

Immunoblot analysis was performed with antiserum C20 (Santa Cruz) reactive with the carboxyl-terminal 20 aa of p130. The p130 protein was readily detected in wild-type extracts and

A

Genomic Locus

Targeting Vector

Targeted Locus

B C D
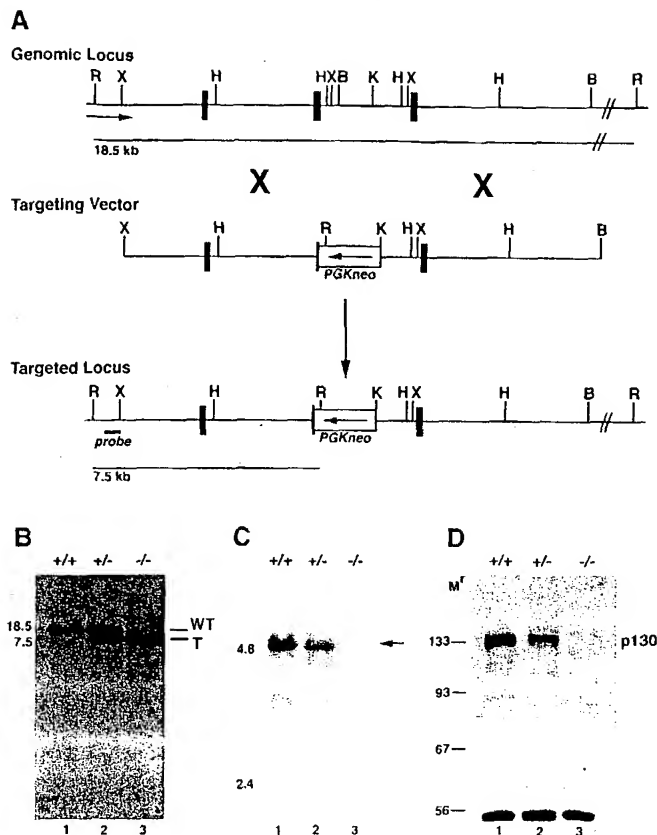
Fig. 1. Targeted disruption of the *p130* gene. (A) Genomic locus, targeting vector and structure of the disrupted *p130* locus with exons depicted as filled boxes. The targeting vector contained 3 kb of 5'- and 7.2 kb of 3'-homologous sequence. The PGK-neo expression cassette was inserted in the opposite transcriptional orientation to *p130* immediately downstream of the aa 106 codon. (B) Southern analysis of *Eco*RV-digested DNA isolated from E11.5 embryos derived from a heterozygous intercross resulted in the predicted restriction length polymorphism. (C) Northern blot analysis of total RNA probed with the full-length mouse cDNA revealed a complete absence of a transcript from the targeted *p130* allele.
(D) Immunoblot analysis with antibody C20 reactive with p130 (Santa Cruz) against tissue lysates indicated that no protein was expressed from the targeted allele. Abbreviations: E, *Eco*RV; X, *Xba*I; H, *Hind*III; B, *Bam*HI; M_r, relative mobility in kD.

reduced levels were observed in *p130*+/- extracts (Fig. 1D, lanes 1 and 2). No detectable product was detected in *p130*-/- lysates (Fig. 1D, lane 3). Moreover, no smaller molecular weight species were apparent in *p130*-/- extracts. Therefore we conclude that disruption of p130 with PGK-neo generated a null allele.

Embryonic lethality in the absence of *p130*

Genotyping of the weaned 3-week-old progeny derived from interbreeding of *p130*+/- mice revealed an absence of *p130*-/- animals. Moreover, inspection of newly delivered litters revealed no increased incidence of non-viable newborn pups. Together, this suggested that *p130*-/- embryos were not being delivered and indicated that *p130*-/- embryos were dying in utero.

Table 1. Viability of embryos derived from *p130*+/- interbreeding

Genotype	Days post coitum					
	9.5	10.5	11.5	12.5	13.5	14.5
Wild type	6	17	15	13	9	5
p130+/-	15	31	23	23	13	14
p130-/-	4	14	9	2	0	0

The F1 *p130*+/- offspring of chimeras bred with Balb/cJ mice were interbred and Cesarean sections performed at different gestational ages. Note, the morning following mating is considered 0.5 days post coitum.

To delineate the gestational stage that *p130*-/- embryos were being lost, Cesarean sections were performed at successive days postcoitum (dpc) following timed matings (Table 1). DNA was isolated from the fetal portion of the placenta and the conceptuses genotyped by Southern analysis. At 9.5 dpc and 10.5 dpc, we observed an approximate Mendelian frequency of 1:2:1 of wild-type, *p130*+/- and *p130*-/- genotypes. However, about 50% of the expected numbers of *p130*-/- embryos were observed on 11.5 dpc, whereas only about 10% of the expected numbers of *p130*-/- embryos were observed on 12.5 dpc. On and after 13.5 dpc, no viable *p130*-/- embryos were detected (Table 1). In addition, we observed that approximately 25% of the conceptuses were non-viable and were undergoing absorption on and after 12.5 dpc. Therefore, we conclude that a null mutation in *p130* in a Balb/cJ genetic background resulted in an embryonic lethal phenotype with embryos dying between embryonic day 11 and 13.

Embryos lacking *p130* display abnormal growth

Inspection of *p130*-/- embryos revealed a disparity in growth that increased with gestational age until 11.5 dpc when the mutant embryo reached approximately 25% of the normal size (Fig. 2A). Mutant embryos at 10.5 dpc exhibited beating hearts with seemingly normal vascularization and distribution of blood. However, mutant hearts displayed an abnormal dilated morphology and appeared to resemble the two-chambered hearts of earlier-stage embryo. More anterior structures appeared normal, for example, 10.5 dpc *p130*-/- embryos exhibited normal brain segmentation, normal elaboration of branchial arches and normal morphology of forelimbs. Posterior structures were reduced and mutant 10.5 dpc embryos failed to form hind limb buds. By 11.5 dpc, the remaining viable mutant embryos had progressed little in development and appeared similar in size to 10.5 dpc *p130*-/- embryos (Fig. 2A).

One possible explanation for the observed growth arrest of *p130*-/- embryos was that function of the placenta was compromised due to abnormal placental development. Importantly, dissection of the placentas from *p130*-/- embryos revealed a normal anatomy and arrangement of extraembryonic blood vessels and membranes. Histological analysis of hematoxylin-stained sections indicated a normal cytomorphology including that of giant cells and placental labyrinth. To assess the extent of apoptosis in *p130*-/- placentas, TUNEL analysis was performed to detect the presence of fragmented DNA in apoptotic bodies (Gavrieli et al., 1992). Importantly, no evidence of apoptosis was detected by TUNEL staining (Fig. 3). Taken together, these data support
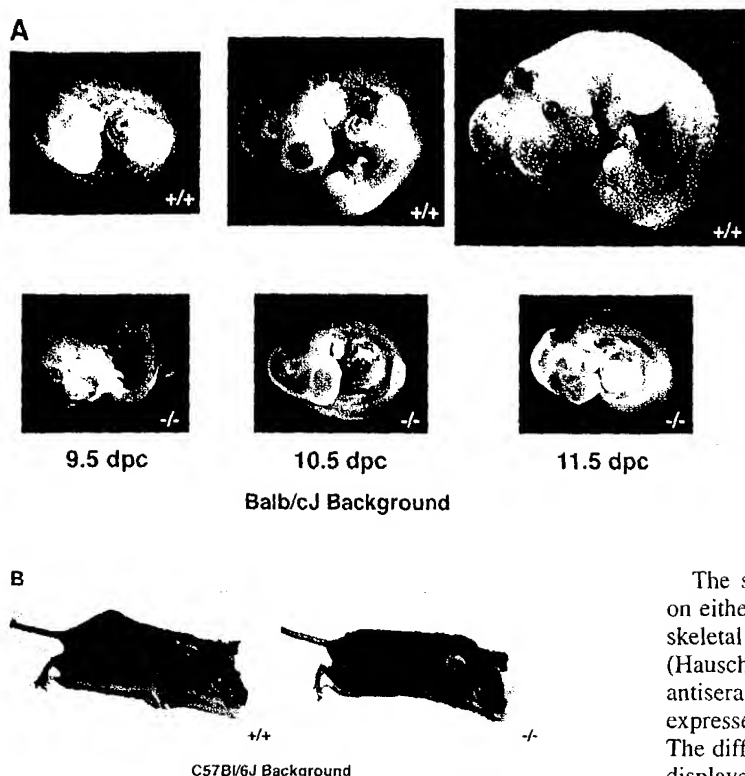
A

9.5 dpc   10.5 dpc   11.5 dpc

Balb/cJ Background

B

+/+   -/-

C57Bl/6J Background

Fig. 2. Embryonic growth deficiency in the absence of p130 is strain-dependent. (A) Wild-type embryos at 9.5 dpc have turned whereas $p130^{-/-}$ embryos were typically observed in the lordotic position and displayed reduced numbers of somites. By 10.5 dpc, $p130^{-/-}$ embryos are about half the normal size of wild-type embryos and displayed normal development of brain structures, however more posterior structures, including the heart and the hind limb region, were underdeveloped. The mutant embryo at 11.5 dpc was strikingly smaller than wild type and appeared arrested at the E10.5 stage. (B) In the C57Bl/6J genetic background, $p130^{-/-}$ mice were viable, fertile and displayed no overt phenotype (see Table 2). Note, 9.5 dpc and 10.5 dpc embryos were photographed before fixation, whereas 11.5 dpc embryos were photographed postfixation.

the conclusion that the growth deficit of $p130^{-/-}$ embryos was not due to placental failure.

Impaired neurogenesis and myogenesis in $p130^{-/-}$ embryos

Histological examination of sections of 10.5 dpc $p130^{-/-}$ embryos revealed some variability in the histological appearance of embryos presumably reflecting their overall viability. A typical 10.5 dpc $p130^{-/-}$ embryo that exhibited a beating heart when delivered is described below. Histological analysis revealed varying degrees of disorganization in neural and dermamyotomal structures, a poorly formed notochord and, in addition, a thin myocardium (Figs 4, 5).

Neuroepithelial cells within the neural tube are the progenitors of motor neurons that differentiate in response to signals from the floor plate of the neural tube (Yamada et al., 1991, 1993). To assess neuronal differentiation, immunohistochemistry was performed with antibody reactive against the LIM-domain transcription factors Isl-1 and Isl-2, expressed in newly born motor and sensory neurons (Ericson et al., 1992; Tsuchida et al., 1994). We observed severely decreased numbers of Isl-1/2-expressing motor neurons within the ventral horn of a somewhat disorganized neural tube and similarly decreased numbers of sensory neurons within a poorly demarcated dorsal root ganglia (compare Fig. 4A and B). Moreover, the neural epithelium in the neural tube entirely failed to elaborate a basement membrane and cells were not organized into layers as in the wild-type neural tube (Figs 4B,D, 5). The floorplate of the neural tube was observed to have undergone marked apoptotic loss at thoracic and lumbar levels (compare Figs 4A and B, 5A and B). However, reduced numbers of neurons were noted at all levels.

The somite-derived dermamyotome, segmentally arranged on either side of the neural tube, forms the first differentiated skeletal muscle of the embryo known as the myotome (Hauschka, 1994). Myogenic differentiation was assessed with antisera reactive to desmin, an intermediate filament protein expressed in skeletal and cardiac muscle (Kablar et al., 1997). The differentiated myotome of wild-type embryos at 10.5 dpc displayed the typical pattern of desmin-expressing myocytes (Fig. 4C). By contrast, the differentiated myotome of $p130$-deficient embryos was composed of very few desmin-expressing skeletal myocytes (compare Fig. 4C and D). The observed reduction in numbers of myotomal myocytes was also found at all levels.

Inspection of 10.5 dpc $p130^{-/-}$ embryos revealed a somewhat dilated myocardium and abnormal cardiac morphology suggestive of a defect in chamber formation (Fig. 2A). To characterize cardiac structure of $p130^{-/-}$ embryos, serial sections were performed through the hearts and
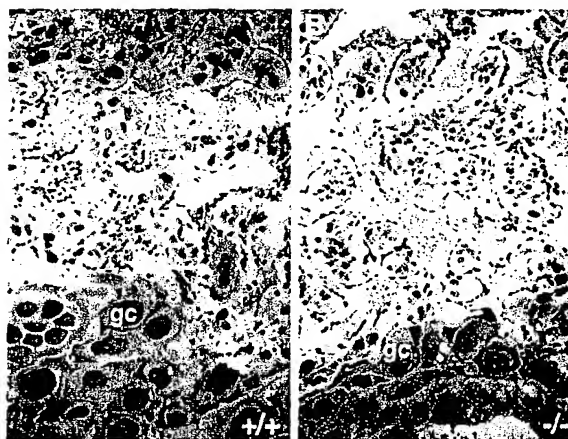

Fig. 3. Normal placental cytomorphology and absence of apoptosis of p130-deficient placentas. The placentas of wild-type (A) and $p130^{-/-}$ (B) embryos were identical in appearance and both contained very few apoptotic bodies. Abbreviations: gc, giant cells; la, labyrinth. Panels were photographed at a magnification of 400×.

Fig. 4. Deficient myogenesis and neurogenesis and associated apoptosis in E10.5 *p130⁻/⁻* embryos. Wild-type embryos (A) contained numerous motor and sensory neurons in the neural tube and dorsal root ganglia as detected with antibody reactive to Isl-1/2. Embryos lacking p130 (B) were reduced in size, displayed a disorganized morphology and contained severely reduced numbers of Isl-1/2-expressing motor and sensory neurons. The normal myotome of wild-type embryos (C) was reduced to a small rudiment in *p130⁻/⁻* embryos (D) as revealed by staining with antibody reactive to desmin. TUNEL analysis revealed low levels of apoptosis in wild-type embryos (E,G) and markedly increased numbers of apoptotic bodies (arrowheads) in the neural tube, dorsal root ganglia and dermamyotome of *p130⁻/⁻* embryos (F,H). Note the absence of the notochord, the disorganized neural floor plate and absence of a basement membrane in the neural tubes of *p130⁻/⁻* embryos (B,F). Abbreviations: nt, neural tube; nc, notochord; mn, motor neurons; drg, dorsal root ganglia; dm, dermamyotome. Panels were photographed at magnification of 200× (A-D), 400× (E-H).
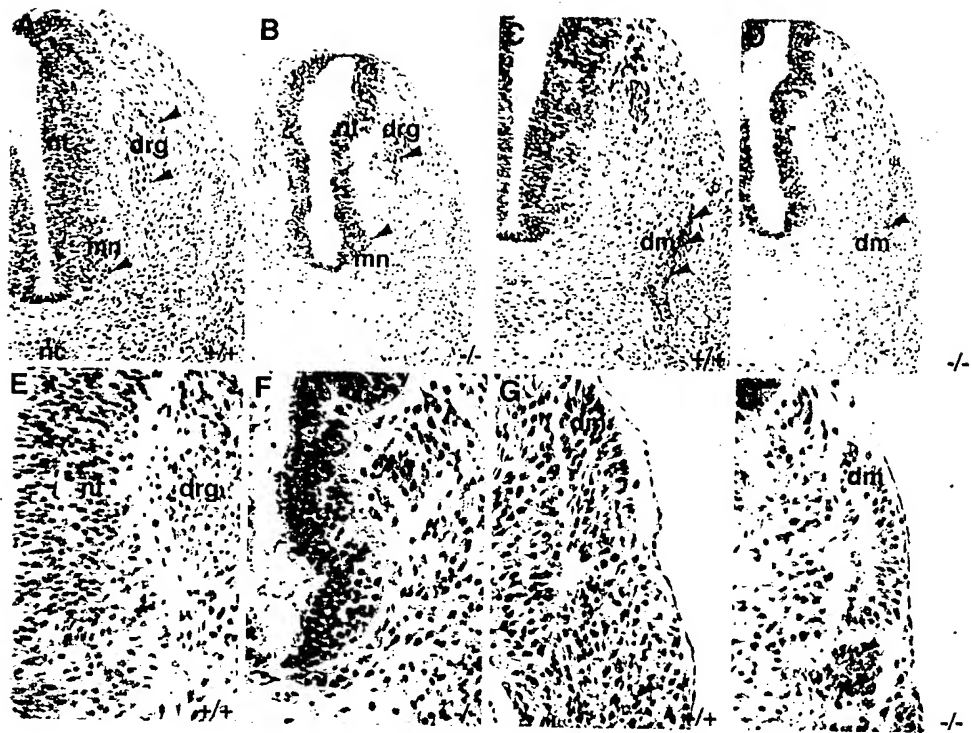
immunohistochemistry was performed with antibody reactive with desmin. The myocardium of *p130⁻/⁻* embryos was poorly developed with a thin wall usually only a single cell in thickness. However, the pericardium and endocardium appeared normal. Examination of serial sections revealed a failure to properly loop and form the four-chambered heart (compare Fig. 6A and B, C and D). Instead, the mutant heart somewhat resembled the two chambered E8.5 heart consisting of the bulbus cordis and the ventricular chamber (Fig. 6).

Increased apoptosis and cellular proliferation in *p130⁻/⁻* embryos

The observed deficiency in neurogenic and myogenic development and presence of numerous subcellular bodies suggested that many cells in *p130⁻/⁻* embryos had undergone programmed death. Therefore, to assess the levels of apoptosis in *p130*-deficient embryos, we performed TUNEL analysis on sectioned material. Wild-type embryos typically contained few apoptotic cells disseminated through the neural tube and dermamyotome (Figs 4E,G, 5A). By contrast, *p130⁻/⁻* embryos contained numerous apoptotic bodies throughout the neural tube and floor plate, and within the epithelial and delaminating portions of the dermamyotome (Figs 4F,H, 5B). In addition,

extensive apoptosis was also observed in the midgut and urogenital ridge, but not the mesonephros (Fig. 6). Moreover, little or no apoptosis was detected in the *p130⁻/⁻* lung bud,

Fig. 5. Loss of floor plate in E10.5 *p130⁻/⁻* embryos. TUNEL analysis revealed low levels of apoptosis in wild-type neural tube (A) and markedly increased numbers of apoptotic bodies (arrowheads) in the neural tube of *p130⁻/⁻* embryos (B). Note the almost complete absence of a floor plate in the *p130⁻/⁻* neural tube. Abbreviations: fp, floor plate; nc, notochord. Panels were photographed at magnification of 400×.

foregut and hepatic primordia (Fig. 7). Note that the morphological development of the midgut, urogenital ridge, mesonephros, lung bud, foregut and liver primordia were at an appropriate stage for 10.5 dpc embryos but appeared abnormal due to markedly reduced cellularity in surrounding structures and poorly elaborated basement membranes. Interestingly, the myocardium of $p130^{-/-}$ embryos, like the wild-type myocardium, contained very few apoptotic nuclei (Fig. 8E,F). Taken together, these data suggest that the absence of $p130$ differentially affects the differentiation or survival of myotomal and neuronal cells versus cardiac myocytes.

Examination of neural structures in the heads of $p130^{-/-}$ embryos suggested that cell survival within the developing central nervous system was severely perturbed in the absence of $p130$. TUNEL analysis of 10.5 dpc $p130^{-/-}$ embryos revealed reduced size and extensive apoptosis in the optic vesicle (Fig. 9E), optic stalk (Fig. 9G), facial acoustic neural crest complex (Fig. 9I) and otic vesicle (Fig. 9K). By contrast, wild-type embryos displayed only moderate numbers of apoptotic bodies in head neural structures (Fig. 9A,D,F,H,J). Interestingly, Rb-deficient embryos display elevated apoptosis and inappropriate proliferation in brain and retinal neurons

(Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992, 1994; Maandag et al., 1994). Therefore, taken together these data suggest that Rb and p130 play important functions in coupling cellular differentiation to cell-cycle control, particularly in the context of neural cell development.

To investigate whether $p130^{-/-}$ embryos exhibited inappropriate proliferation, we performed immunohistochemistry with antibody PC10 reactive with proliferating cell nuclear antigen (PCNA). Replicating cells

Fig. 6. Increased apoptosis in the urogenital ridge and midgut in $p130^{-/-}$ 10.5 dpc embryos. TUNEL analysis revealed low levels of apoptosis in wild-type embryos (A,C,E) and markedly increased numbers of apoptotic bodies (arrowheads) in the urogenital ridge (B,D) and midgut (B,F) of $p130^{-/-}$ embryos. Abbreviations: a, midline dorsal aorta; dm, dermamyotome; g, midgut; m, mesonephric duct/vesicle; nt, neural tube; u, urogenitol ridge. Panels were photographed at magnification of 200× (A-D) and 400× (C-F).
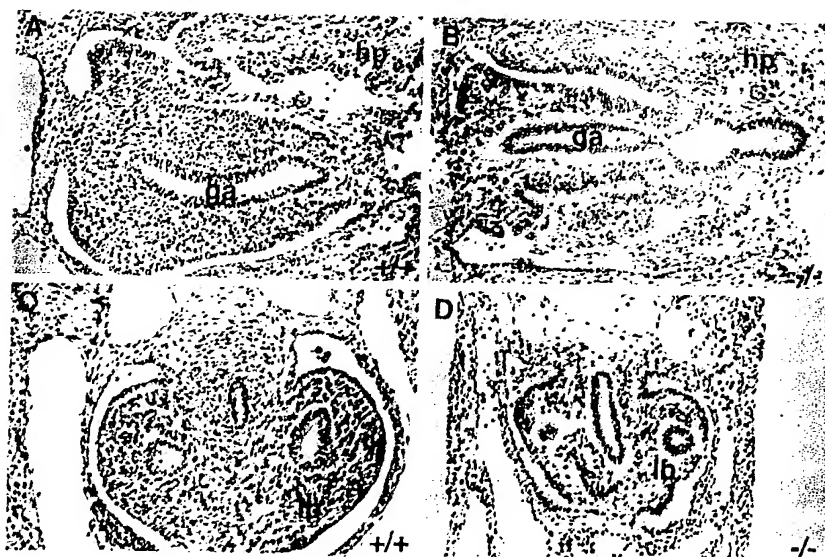

Fig. 7. Absence of apoptosis in lung bud, foregut and hepatic primordia in E10.5 $p130^{-/-}$ embryos. TUNEL analysis revealed negligible levels of apoptosis in both wild-type (A,C) and $p130^{-/-}$ (B,D) lung bud, foregut and hepatic primordia. Abbreviations: lb, lung bud; ga, gastric dilatation of foregut; hp, hepatic/bilary primordia. Panels were photographed at magnification of 400×.

Fig. 8. Abnormal cardiogenesis in *p130⁻/⁻* embryos. Immunochemistry with antibody reactive with desmin revealed the four-chambered myocardium of wild-type embryos (A,C). By contrast, the myocardium of *p130⁻/⁻* embryos (B,D) was poorly developed and serial sections indicated the presence of two-chambers, the bulbus cordis and the ventricular chamber. TUNEL analysis did not reveal any significant apoptosis in wild-type (E) or mutant (F) hearts. Abbreviations: m, myocardium; pc, pericardium; ra, right atria; la, left atria; rv, right ventricle; lv, left ventricle. Panels were photographed at magnification of 100× (A,D) and 400× (B,C,E,F).
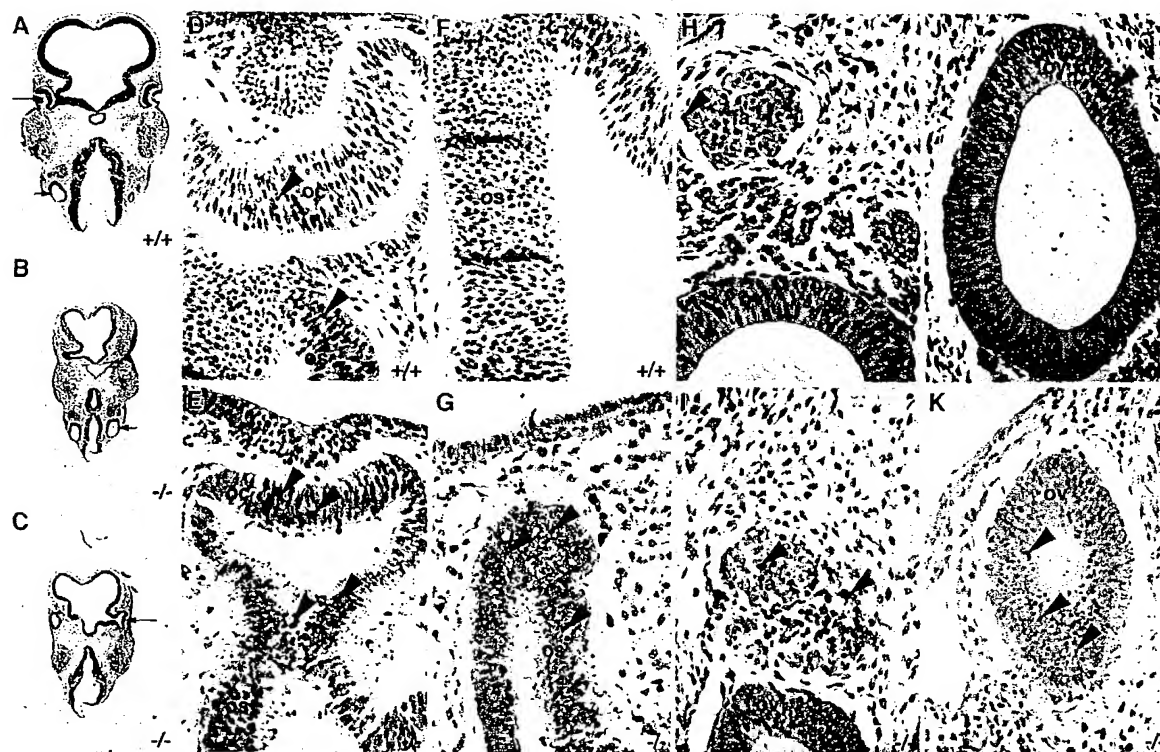
Fig. 9. Increased apoptosis and poor differentiation of head neural structures in the absence of p130. TUNEL analysis of 10.5 dpc wild-type embryos (A) revealed moderate numbers of apoptotic bodies in the optic vesicles (D), optic stalks (F), facial acoustic neural crest complexes (H) and otic vesicles (J). The head neural structures of *p130⁻/⁻* embryos (B, C) displayed reduced size and extensive apoptosis in the optic vesicles (E), optic stalks (G), facial acoustic neural crest complexes (I) and otic vesicles (K). Note the absence of a basement membrane lining the neural structures of *p130⁻/⁻* embryos. In A-C, long arrow denotes optic vesicle and stalk, and short arrow denotes otic vesicle. In the remainder of the panels, arrowheadss indicate TUNEL-positive cells. Abbreviations: oc, optic cup; os, optic stalk; fa, facio-auditory pre-ganglion complex; ov, otic vesicle. Panels were photographed at 400× magnification with the exception of A-C, at 25×.
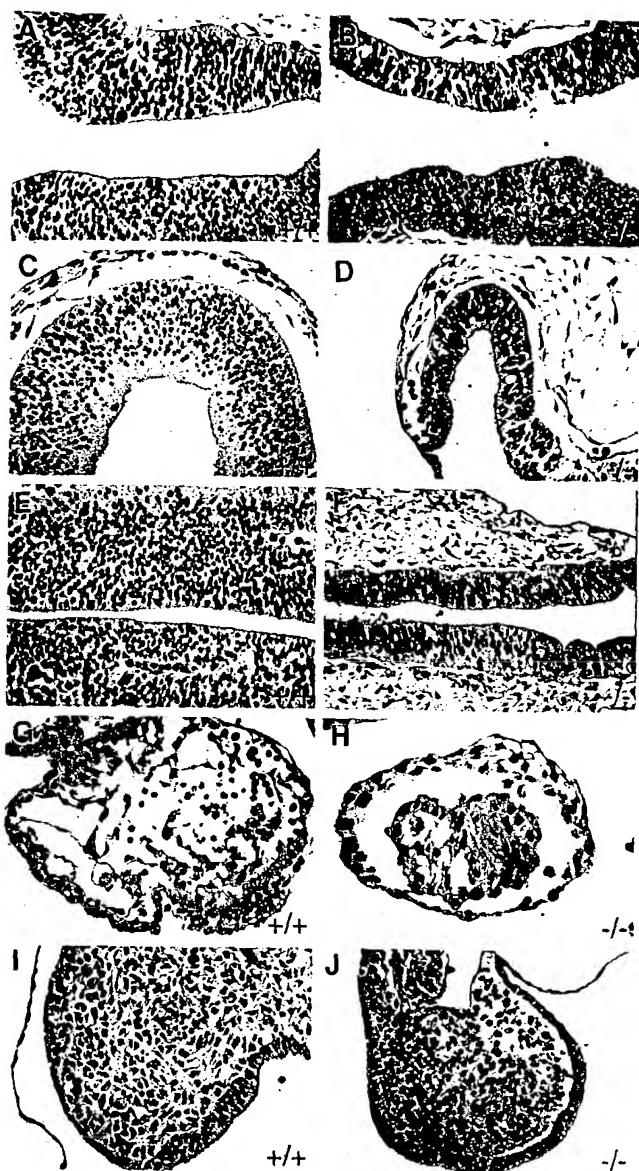
Fig. 10. Detection of increased cellular proliferation by PCNA-expression. Immunohistochemistry of sectioned E10.5 $p130^{-/-}$ embryos with antibody PC10 revealed over a 2-fold increase in numbers of PCNA-expressing cells as revealed by nuclear staining in the telencephalon (A,B), diencephalon (C,D) and the neural tube (E,F). By contrast, $p130^{-/-}$ cardiac muscle contained 1.6-fold fewer proliferative cells (G,H) and no difference in the proportion of PCNA-expressing cells was observed between mutant and wild-type branchial arches (I,J). Panels were photographed at 400× magnification.

express PCNA at high levels during S-phase thus immunodetection of PCNA in tissues allows a determination of relative mitotic activity (Megeney et al., 1996). In 10.5 dpc $p130^{-/-}$ embryos, the numbers of PCNA-expressing cells were increased 2.1-fold in the telencephalon (Fig. 10A,B), 2.6-fold in the diencephalon (Fig. 10C,D) and 2.1-fold in the neural tube (Fig. 10E,F). By contrast $p130^{-/-}$ cardiac muscle contained 1.6-fold fewer PCNA-expressing cells (Fig. 10G,H), and no difference in the proportion of PCNA-expressing cells

Table 2. Genetic background specifies the penetrance of the $p130^{-/-}$ phenotype

Genotype	Intercross		
	Chimera × Balb/cJ ↓ $F_1^{+/-} \times F_1^{+/-}$*	$F_1^{+/-} \times$ Balb/cJ ↓ $B_1^{+/-} \times B_1^{+/-}$‡	$F_1^{+/-} \times$ C57BL/6J ↓ $B_1^{+/-} \times B_1^{+/-}$§
Wild type	42	12	21
$p130^{+/-}$	78	28	39
$p130^{-/-}$	0	0	24

*The F_1 $p130^{+/-}$ progeny of the founding chimeras bred with Balb/cJ mice when interbred yielded no viable $p130^{-/-}$ pups.

‡The B_1 $p130^{+/-}$ mice derived from an F_1 $p130^{+/-}$ × Balb/cJ mating when interbred also failed to produce $p130^{-/-}$ mice.

§The B_1 $p130^{+/-}$ mice, derived from a F_1 $p130^{+/-}$ × C57BL/6J mating, when interbred generated litters that contained viable and fertile $p130^{-/-}$ mice that displayed an apparently normal phenotype (see Fig. 2B).

was observed between mutant and wild-type branchial arches (Fig. 10I,J). Therefore, a tissue-specific correlation between the presence of inappropriate proliferation and increased apoptosis was evident in $p130^{-/-}$ embryos.

The *p130* mutant phenotype is strain dependent

The relatively normal phenotype of the $p130^{-/-}$ mice previously described in a mixed 129/Sv:C57BL/6J genetic background (Cobrinik et al., 1996) and the embryonic lethal phenotype of $p130^{-/-}$ mice in an enriched Balb/cJ background suggested that the penetrance of the $p130^{-/-}$ phenotype was dependent on second site modifier genes. To test this hypothesis, we bred F_1 $p130^{+/-}$ mice, the progeny of the founding chimeras and Balb/cJ mice, with either C57BL/6J or Balb/cJ mice. The resulting B_1 $p130^{+/-}$ mice were then interbred to generate $p130^{-/-}$ mice. The B_1 $p130^{+/-}$ mice derived from the F_1 $p130^{+/-}$ × C57BL/6J cross have one set of C57BL/6J chromosomes and a second set composed of a mixture of Balb/cJ and 129/Sv chromosomes. The 129/Sv chromosomes are derived from the embryonic stem cells. The B_1 $p130^{+/-}$ mice derived from the F_1 $p130^{+/-}$ × Balb/cJ cross have one set of Balb/cJ chromosomes and a second set composed of an undefined mixture of Balb/cJ and 129/Sv chromosomes. Thus, such crosses allow an assessment of the contribution of Balb/cJ and C57BL/6J genetic backgrounds to the penetrance of the phenotype. However, these experiments do not directly assess the contribution of the 129/Sv genetic background to the penetrance of the phenotype.

As described above, $p130^{-/-}$ animals derived from an F_1 $p130^{+/-}$ × F_1 $p130^{+/-}$ mating displayed 100% penetrance of the lethal phenotype (see Table 2, 1st column). In a small proportion of F_2 $p130^{-/-}$ × F_2 $p130^{-/-}$ matings, we observed litters that contained a mixture of runted and normal-sized F_3 $p130^{-/-}$ mice suggesting that multiple recessive second-site modifier genes were segregating in the population. Interbreeding of B_1 $p130^{+/-}$ mice derived from a F_1 $p130^{+/-}$ × Balb/cJ mating gave rise to $p130^{-/-}$ mice that also exhibited a 100% penetrance of the phenotype (Table 2, 2nd column). By contrast, interbreeding of B_1 $p130^{+/-}$ mice derived from a F_1 $p130^{+/-}$ × C57BL/6J mating gave rise to $p130^{-/-}$ mice that were viable and fertile, and displayed no detectable phenotype (Fig. 2B). Taken together, these data suggest that the C57BL/6J genetic background suppressed the $p130^{-/-}$ embryonic lethal phenotype apparent on a Balb/cJ genetic background (Table 2,

3rd column). Therefore, we conclude that multiple second-site modifier genes exist that have an epistatic relationship with p130.

DISCUSSION

We have generated a null allele of *p130* by gene targeting in mice and crossed the mutant allele into Balb/cJ and C57BL/6J strains of mice. Embryos lacking *p130* in a genetic background enriched for Balb/cJ were reduced in size and died between embryonic stages E11 and E13. Immunohistochemistry with Isl-1 antibody revealed profoundly reduced numbers of motor neurons in the spinal cord and sensory neurons in the dorsal root ganglia. In addition, immunohistochemistry with antibody reactive to desmin similarly indicated markedly reduced numbers of differentiated myocytes within the myotome. The hearts of mutant embryos displayed unusually thin walls and appeared delayed in development. TUNEL analysis indicated the presence of numerous apoptotic bodies in many tissues including the neural tube, dermamyotome and brain, but not in the heart or the histologically normal placenta. Immunohistochemistry with antibody reactive with PCNA revealed increased cellular proliferation in the neural tube and the brain, and decreased proliferation in the heart. Importantly, following a backcross to C57BL/6J mice, $p130^{-/-}$ animals were derived that were phenotypically normal. These data clearly indicate that p130 plays an essential role in development, but in a strain-dependent manner.

Embryos deficient for p130 contained low numbers of Isl-1, expressing neurons in the neural tube and low numbers of desmin-expressing myocytes in the dermamyotome. This deficiency was correlated with the presence of reduced notochord and floor plate structures in the trunk, together with increased levels of apoptosis. Several contributing mechanisms can be proposed to functionally explain the embryonic lethal phenotype in the absence of p130 in a genetic background enriched for Balb/cJ. For example, patterning and morphogenesis may be perturbed following loss of key structures during development and cellular differentiation. Alternatively, cell survival may be detrimentally affected because of a unique function of p130 in withdrawal from the cell cycle or in enforcing terminal differentiation.

Apoptotic loss of structures like the notochord during the development of $p130^{-/-}$ embryos could contribute significantly to the embryonic phenotype. For example, the determination of progenitors of motor neurons is regulated in part by signals from the notochord and floor plate of the neural tube (Yamada et al., 1991, 1993). Sonic hedgehog (Shh) is expressed in the notochord and the floor plate in the trunk where it functions to induce the progenitors of motor neurons. These progenitors, situated in the ventricular epithelium of the ventral neural tube, are induced to migrate laterally and to differentiate and settle in a single continuous primary motor column (Tanabe et al., 1995). Additionally, Shh, expressed in the floor plate and the notochord, and Wnt family members, expressed in the dorsal neural tube, have been suggested to combinatorially activate myogenesis in the somite (Munsterberg et al., 1995). Wnts positively stimulate myogenesis in the somite whereas Shh is believed to activate Noggin expression in the dorsal somite, inhibiting the repression of myogenesis by lateral-plate-

derived BMP4 (Hirsinger et al., 1997; Marcelle et al., 1997; Reshef et al., 1998). Therefore, it is interesting to speculate that loss of structures such as the floorplate in $p130^{-/-}$ embryos may contribute to the severity *p130*-mutant phenotype.

A loss-of-function mutation in *p130* may also result in cell-autonomous deficits in cellular differentiation. For example, the reduced neurogenesis and myogenesis observed in $p130^{-/-}$ embryos may reflect a global perturbation of patterning due to specific requirements for p130 in directly negatively regulating proteins with paired-like homeodomains that play key developmental roles (Wiggan et al., 1997). Alternatively, appropriate withdrawal from the cell cycle and terminal differentiation may be detrimentally affected due to an important regulatory role played by the formation of specific E2F/p130 complexes (Muller, 1995; Whyte, 1995). For example, myoblasts contain free E2F as well as E2F complexed with p107 and to a lesser degree p130, but not Rb, whereas differentiated myocytes primarily contain E2F complexed with p130, but not p107 or Rb (Corbeil et al., 1995; Shin et al., 1995). In differentiated myocytes, E2F complexes are primarily composed of E2F-4/p130 and formation of this complex has been suggested to be a necessary event in terminal differentiation (Puri et al., 1997; Shin et al., 1995). Similarly, formation of analogous E2F/p130 complexes has been observed during both neuronal and cardiomyocyte differentiation (Flink et al., 1998; Raschella et al., 1997). Therefore, the low numbers of cells expressing Isl-1 or desmin in $p130^{-/-}$ embryos may reflect an important and unique role for p130 in withdrawing from the cell cycle or enforcing terminal differentiation. Clearly, the presence of markedly increased numbers of PCNA-expressing cells in tissues containing increased numbers of apoptotic cells supports this later hypothesis.

In *Rb*-deficient embryos, cells continue to replicate in regions of the central and peripheral nervous system that normally contain only postmitotic cells with many of the neurons undergoing apoptosis shortly after entering an ectopic S-phase (Lee et al., 1994). Apoptosis in the nervous system of $Rb^{-/-}$ embryos is p53-dependent and correlates with increased levels of E2F, cyclin E and p21 (Macleod et al., 1996). In muscle, lack of Rb similarly results in apoptotic loss of inappropriately proliferating cells that fail to undergo terminal differentiation (Wang et al., 1997; Zacksenhaus et al., 1996). Heterozygous *Rb* mice develop lens cataracts due to loss-of-homozygosity in *Rb*, in which cells are poorly differentiated, are highly proliferative and undergo very high rates of apoptosis. By contrast, heterozygous Rb mice bred into a p53-homozygous mutant background exhibit overt lens hyperplasias with no associated apoptosis (Morgenbesser et al., 1994). Similarly, transgenic mice expressing human papilloma virus type 16 (HPV-16) E7 in retinal cells exhibit very high rates of retinal cell apoptosis. However, expression of both E7 and E6 transgenes, or the E7 transgene in p53-mutant mice induces retinal tumors with a reduction or absence of associated apoptosis (Howes et al., 1994; Pan and Griep, 1994). HPV-16 E7-protein binds all Rb-family members suggesting that the failure of retinoblastomas to form in targeted Rb-mutant mice is a consequence of functional redundancy amongst the Rb-family.

Inappropriate activation of E2F in a wide variety of cell types leads to p53-enhanced apoptosis (Hiebert et al., 1995;

Phillips et al., 1997; Qin et al., 1994; Shan and Lee, 1994). Moreover, Rb and p130 appear to induce G$_1$ arrest via biochemically distinct mechanisms involving either E2F-1 or E2F-4 (Vairo et al., 1995). Therefore, generation of compound p130$^{-/-}$;p53$^{-/-}$ embryos in a Balb/cJ genetic background may elucidate whether the observed widespread apoptosis is p53-dependent as well as potentially allow partial rescue of the phenotype.

We have also derived a targeted null mutation in p107 and have bred the mutant allele into either Balb/cJ or C57BL/6J genetic backgrounds. We observed that p107$^{-/-}$ embryos in an enriched Balb/cJ background are viable and fertile but exhibit diathetic myeloid metaplasia, a severe postnatal growth deficiency and an accelerated cell cycle (LeCouter et al., 1998). By contrast, p107$^{-/-}$ mice in a C57BL/6J background display no apparent phenotype (LeCouter et al., 1998; Lee et al., 1996). These data strongly support our interpretation that second-site modifier genes exist that effect the penetrance of null mutations in both p130 and p107.

Mice carrying targeted null mutations (for example in IGF-1, fibronectin, EGFR, CFTR, TGFβ1, TGFβ3 and β1-adrenergic receptor) can display highly variable penetrance of phenotype on different genetic backgrounds (Bonyadi et al., 1997; George et al., 1993; Liu et al., 1993; Proetzel et al., 1995; Rohrer et al., 1996; Rozmahel et al., 1996; Sibilia and Wagner, 1995; Threadgill et al., 1995). Clearly, these observations underscore the significance of second-site modifier genes when characterizing null mutations. The molecular basis for the penetrance of the p130/- phenotype on C57BL/6J versus Balb/cJ backgrounds remains to be established. Nevertheless, the breeding data is consistent with the existence of multiple modifier alleles representing either recessive loss-of-function mutations in the C57BL/6J background, dominant gain-of-function mutations in the Balb/cJ background, or a mixture of both (Table 2). Alternatively, our data does not rule out the possibility that heterozygosity at some modifier alleles contributes to the observed phenotype. In addition, our experiments do not directly assess the role played by the ES-derived 129/Sv chromosomes segregating in the different offspring. However, genetic analysis should allow a resolution of this issue. Currently, we are performing microsatellite analysis to accurately determine the number of modifying genes and to map their approximate locations. Clearly, the molecular identification of genes epistatically interacting with p130 and p107 will further our understanding of the regulatory pathways within which Rb-family members operate.

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Role of the Common Cytokine Receptor γ Chain in Cytokine Signaling and Lymphoid Development

Warren J. Leonard[1], Elizabeth W. Shores[2] & Paul E. Love

INTRODUCTION

X-linked severe combined immunodeficiency (XSCID) is an inherited disease in which patients exhibit profoundly diminished cell-mediated and humoral immunity. This disease results from mutations in the common cytokine receptor γ chain, γ_c. In order to understand more about the role of γ_c in lymphoid development, we have analyzed mice in which the γ_c gene was specifically inactivated by homologous recombination. These mice exhibit a wide range of interesting immunological abnormalities, some shared by humans with XSCID and some that are different. The differences are indicative of variations in lymphoid development between humans and mice, and potentially will allow new insights into the roles of γ_c-dependent cytokines in both species. Moreover, the mutant ($\gamma_c^{-/Y}$) mice provide a valuable animal model of γ_c deficiency and therefore represent potential targets for reconstitution by gene therapeutic approaches.

X-LINKED SEVERE COMBINED IMMUNODEFICIENCY: IDENTIFICATION OF THE MOLECULAR DEFECT

Severe combined immunodeficiency diseases represent a spectrum of disorders (reviewed in Leonard et al. 1994, Leonard, in press), with XSCID accounting for ap-

[1]Laboratory of Molecular Immunology, National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20892, U.S.A.; [2]Division of Hematologic Products, Center for Biologics Evaluation and Research, FDA, Bethesda, MD 20892, U.S.A.; [3]Laboratory of Mammalian Genes and Development, National Institute of Child Health and Human Development, NIH, Bethesda, MD 20892, U.S.A.
Correspondence to: Dr. Warren J. Leonard, Bldg. 10, Rm. 7N244, NHLBI, Bethesda, MD 20892-1674

proximately half of all cases of SCID (Conley 1992). Affected males exhibit pro-
foundly diminished numbers of T cells, explaining the lack of cell-mediated immu-
nity. B-cell numbers are normal or even increased, but are nonfunctional. The lack
of B-cell function has been assumed to result in part from a lack of T-cell help
since the addition of normal T cells *in vitro* can promote at least some B-cell re-
sponses (Conley 1992). However, an intrinsic B-cell defect is also indicated by the
non-random X-inactivation patterns observed in the mature B cells of XSCID car-
rier females (Conley 1992). XSCID patients also lack natural killer (NK) cells. In
contrast to these lymphoid abnormalities, other blood cells are represented in nor-
mal numbers and exhibit normal function.

The discovery of the molecular defect in XSCID came not from studies tar-
geted to that end, but rather from basic studies on the IL-2 receptor. This receptor
is known to contain at least three subunits, the α (Leonard et al. 1982, Leonard et
al. 1984, Nikaido et al. 1984, Cosman et al. 1984), β (Sharon et al. 1986, Tsudo et
al. 1986, Teshigawara et al. 1987, Hatakeyama et al. 1989) and γ (Takeshita et al.
1992) chains. These three chains together are responsible for forming three
classes of IL-2 receptors. Whereas intermediate affinity receptors (β + γ chains)
are expressed on resting lymphocytes, high affinity (α + β + γ chains) and low af-
finity (α chains without β or γ) receptors are found on activated T cells (Leonard
et al. 1994, Taniguchi 1995, Leonard, in press). The high and intermediate affinity
receptors are capable of transducing IL-2 signals, whereas low affinity receptors
cannot. Corresponding to this observation, it is interesting that both β and γ
chains (the shared components of high and intermediate affinity receptors) are
members of the cytokine receptor superfamily (Bazan 1990), whereas α is not.
Experiments using chimeric receptor constructs indicate that heterodimerization
of the cytoplasmic domains of β and γ is necessary and sufficient for signaling
(Nakamura et al. 1994, Nelson et al. 1994).

Analysis of the γ chain gene revealed that it was located on the X chromosome
at position Xq13 (Noguchi et al. 1993c) previously established to be the locus of
XSCID (the SCIDX1 locus)(de Saint Basile et al. 1987). Sequencing of DNA
prepared from EBV infected cell lines from XSCID patients subsequently estab-
lished that the γ chain gene was defective in XSCID (Noguchi et al. 1993c). A
large number of patients with XSCID have now been studied (reviewed in Leon-
ard, in press). The range of identified mutations include a number of premature
stop codons, insertions, deletions and splice junction defects of such a nature that
even if the γ chain mRNA and protein product were stably produced, functional
alterations would be expected in the protein. In addition, a number of point muta-
tions have been found that result in single amino acid changes (Puck et al. 1993,
DiSanto et al. 1994a, 1994b, Ishii et al. 1994, Russell et al. 1994, Clark et al.
1995). In some cases, amino acid alterations in the extracellular domain have
been demonstrated to inhibit IL-2 binding, whereas mutations in the cytoplasmic
domain interfere with signal transduction. Thus, a number of naturally occurring
mutations have helped to clarify the functional domains of the γ chain protein.
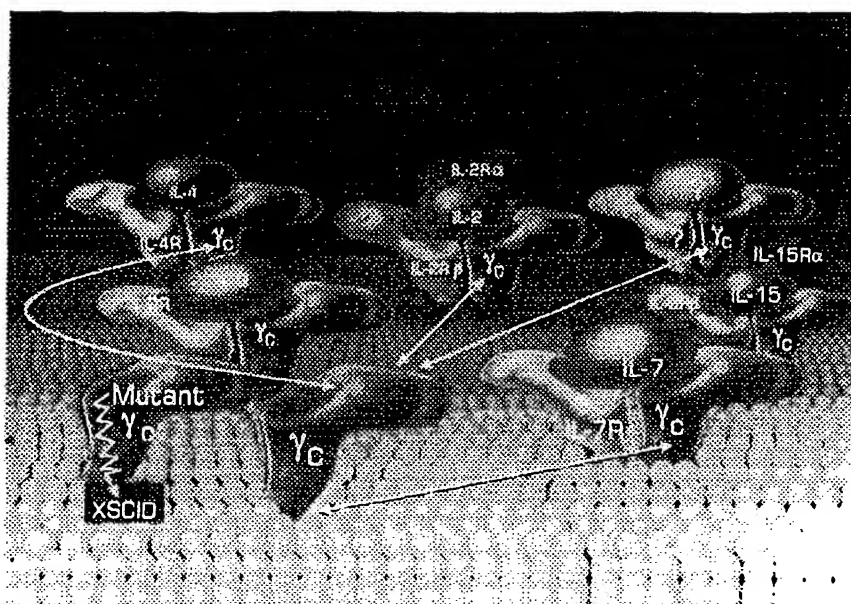
Figure 1. Schematic of the common cytokine receptor γ chain (γ$_c$) as a component of the IL-2, IL-4, IL-7, IL-9 and IL-15 receptors. In males, XSCID results from mutations in γ$_c$.

A COMMON CYTOKINE RECEPTOR SUBUNIT

One of the puzzling features of the discovery that defects in γ chain caused XSCID in humans was that, in contrast to the profound block in T-cell development observed in XSCID patients, mice (Schorle et al. 1991) and humans (Pahwa et al. 1989, Weinberg & Parkman 1990) lacking IL-2 expression exhibit normal T-cell development. In other words, mutations in a component of the IL-2 receptor yielded defects more severe than those found associated with IL-2 deficiency. This observation led to the hypothesis that the γ chain was a component of other cytokine receptors as well (Noguchi et al. 1993), consistent with the precedents that the hematopoietic cytokines IL-3, IL-5 and GM-CSF all share a common β chain, β$_c$ (Miyajima et al. 1992), and that another set of cytokines all share the IL-6 receptor signal transducer gp130 (Taga & Kishimoto 1995). Although the γ chain was initially hypothesized (see Leonard et al. 1994 for the rationale underlying this hypothesis) and confirmed (Kondo et al. 1993, Russell et al. 1993, Noguchi et al. 1993b, Kondo et al. 1994) to be a component of the IL-4 and IL-7 receptors, it is now also known to be a component of the IL-9 (Russell et al. 1994, Kimura et al. 1995) and IL-15 (Giri et al. 1994) receptors as well. Since the γ chain is shared by multiple cytokine receptors (Figure 1), it is now denoted as the common cytokine receptor γ chain, γ$_c$, in keeping with the nomenclature adopted for the hematopoi-

etic cytokines (Miyajima et al. 1992). The overall clinical features of XSCID can ostensibly be accounted for by the simultaneous inactivation of five cytokine systems in patients with this disease. In all likelihood, inactivation of IL-7 signaling is primarily responsible for the lack of T-cell development in this disease (Leonard 1994, Leonard, in press), particularly in view of the phenotype of IL-7 deficient mice (von Freeden-Jeffry et al. 1995).

JANUS FAMILY TYROSINE KINASES JAK1 AND JAK3

As noted above, heterodimerization of IL-2Rβ and γ_c is sufficient to trigger cell proliferation. To understand this phenomenon, it was necessary to identify the essential signaling molecule(s) that associate with each chain. It was striking that stimulation of activated T cells or NK cells by IL-2 rapidly induced the tyrosine phosphorylation of Jak1 and Jak3, two different Janus family tyrosine kinases (Johnston et al. 1994, Witthuhn et al. 1994). An analysis of protein–protein interactions revealed that Jak1 associates with IL2Rβ and Jak3 associates with γ_c (Boussiotis et al. 1994, Russell et al 1994, Miyazaki et al. 1994). Moreover, Jak3 can also interact with IL-2Rβ following IL-2 stimulation (Russell et al. 1994). The importance of the γ_c-Jak3 interaction was established by the identification of a family pedigree with an X-linked immunodeficiency in which there was a single amino acid change in γ_c (Leu 271 mutated to Gln)(Schmalstieg et al. 1995, Russell et al. 1994). This mutation was shown to greatly diminish the ability of γ_c to interact with Jak3 (Russell et al. 1994). A particularly exciting aspect of this finding was that affected individuals in this family have a moderate rather than severe form of immunodeficiency; therefore, the partial loss of Jak3 association corresponded to the partial loss of immune function. These data suggest that XSCID may result from mutations of γ_c that directly or indirectly prevent the activation of Jak3 (i.e. either by inhibiting cytokine binding or by preventing γ_c-Jak3 association). Moreover, some autosomal recessive cases of SCID that are clinically and immunologically indistinguishable from XSCID have been found to result from mutations in Jak3 (Macchi et al. 1995, Russell et al. 1995).

DEVELOPMENT OF A MURINE MODEL OF γ_c-DEFICIENCY

Given the profound abnormalities in humans with XSCID, we wished to develop a murine model of γ_c deficiency on the assumption that such animals would (1) allow more detailed analysis of the role of γ_c in lymphoid development, (2) clarify the similarities and differences in γ_c function in humans and mice, (3) serve as a murine model for potential reconstitution of γ_c in distinct lineages, (4) allow structure-function relationships for γ_c, and (5) serve as an animal model of XSCID, thereby facilitating the evaluation of gene therapeutic approaches. Successful gene therapy of mice using already established γ_c-expressing viruses (Qazilbash et al. 1995) could increase the rationale for progressing to gene therapy in humans. A

naturally occurring form of XSCID in dogs (Henthorn et al. 1994, Somberg et al. 1994) may also be valuable in this regard.

CHARACTERIZATION OF THE MURINE γ_c GENE, PREPARATION OF A TARGETING VECTOR, AND GENERATION OF γ_c-DEFICIENT MICE

The murine γ_c gene, like the human gene, is located on the X chromosome; in mice, γ_c maps between *Zfx* and *Plp* (Cao et al. 1993). The murine cDNA has 80% amino acid identity to human γ_c (Cao et al. 1993). The entire gene was cloned and sequenced (Cao et al. 1995). As expected, like the human γ_c gene (Noguchi et al. 1993a), the murine gene is divided into eight exons. A sequence-replacement targeting vector was prepared by standard methodology (Tybulewicz et al. 1991), the vector was transfected into embryonic stem (ES) cells, and homologous recombination achieved (Cao et al. 1995). Because γ_c is located on the X chromosome and the ES cells are of male lineage, a single recombination event was sufficient to create a γ_c-null cell line. The ES cells were injected into C57BL/6 blastocysts, chimeric mice were generated, and germline transmission was achieved. Mating of heterozygous ($\gamma_c^{+/-}$) females with wild-type males or of heterozygous females with $\gamma_c^{-/Y}$ males yielded progeny with the expected ratios based on Mendelian genetics (Cao et al. 1995). In an independent study, $\gamma_c^{-/Y}$ mice were also generated by DiSanto et al. (1995) using a cre-loxP recombination approach.

GROSS AND MICROSCOPIC ANALYSIS OF $\gamma_c^{-/Y}$ MICE

$\gamma_c^{-/-}$ females, $\gamma_c^{+/-}$ females, and $\gamma_c^{-/Y}$ males were indistinguishable in gross appearance from wild-type littermates. Housed under SPF conditions, γ_c-deficient mice that have been followed for up to 7 months appear healthy (our unpublished observations). However, three mice have developed a wasting syndrome characterized by runted appearance, matted hair, and failure to thrive (see below).

At necropsy, heterozygous females were indistinguishable from wild-type males and females, consistent with the normal phenotype of human XSCID carrier females. In XSCID carrier females, strictly nonrandom X-inactivation patterns are observed in T cells, NK cells, and mature B cells (reviewed in Conley 1992, Puck 1993, Leonard et al. 1994); indicating that only those cells in which the mutant X was inactivated could mature and terminally differentiate. Thus, the levels of γ_c in cells of carrier females are normal, equivalent to individuals who have wild-type γ_c at both alleles. Presumably, the same conditions operate in $\gamma_c^{+/-}$ female mice.

The $\gamma_c^{-/Y}$ males examined were all abnormal, but the phenotype differed to some extent, depending on the age of the mice. These data are largely from Cao et al. (1995) and are summarized in Table I. The youngest mice analyzed (3-week-old mice) had small thymuses and small spleens, but otherwise had grossly

TABLE I

Properties of γ_c knockout mice

1. Small hypoplastic thymuses with increased CD4:CD8 ratio.
2. Small hypoplastic spleens at 3 weeks of age but then increasing in cellularity and size. Increased CD4:CD8 ratio.
3. Greatly diminished conventional B cells in bone marrow and spleen but detectable peritoneal B-1 cells.
4. Absent peripheral lymph nodes. Inactive follicles in mesenteric nodes.
5. No gut-associated lymphoid tissue.
6. No $\gamma\delta$ cells in skin, gut, or thymus.
7. No NK cells.
8. Striking γ_c-independent proliferation in thymus.
9. Readily detectable IgM levels; other Ig levels severely diminished.

normal chest and abdominal cavity organs. The slightly older animals that have been examined, ranging in age from 4 to 9 weeks of age, also had small thymuses, but surprisingly, the spleens in these animals were increased in size. Microscopic examination of $\gamma_c^{-/Y}$ thymuses revealed marked lymphoid hypoplasia with an indistinct corticomedullary junction (Cao et al. 1995). Hassall's corpuscles and a cortical rim of lymphocytes could be identified and basic thymic architecture was retained, in contrast to what is seen in SCID/NCR mice. At 3 weeks of age, the spleens were remarkable for their diminished white pulp and lymphocyte hypoplasia. In contrast, the red pulp was indistinguishable from that of normal littermates. However, with increasing age, the spleens increased in size and cellularity.

Although lymph nodes were not grossly observed in $\gamma_c^{-/Y}$ mice, at necropsy, a mesenteric node was identified in a $\gamma_c^{-/Y}$ mouse (Cao et al. 1995). The node was much smaller than mesenteric nodes in normal sibling mice; moreover, the single lymphoid follicle identified lacked a germinal center.

Remarkably, no gut-associated lymphoid tissue could be identified in either the small or large intestine of $\gamma_c^{-/Y}$ mice. Significantly, all of the $\gamma_c^{-/-}$ and $\gamma_c^{-/Y}$ mice necropsied to date (n=13) exhibited enlarged spleens and thickening of the large bowel. Histological examination of the bowel revealed proliferative typhlitis (inflammation of the cecum) and colitis, with an increase in crypt depth. Intestinal intraepithelial lymphocytes (iIELs) were notably absent, but abundant mononuclear infiltrates were observed in the lamina propria. Inflammatory bowel disease (IBD) has also been reported in several other immunodeficient lines of mice generated by gene targeting, including mice lacking IL-2 (Sadlack et al. 1993), IL-10 (Kuhn et al. 1993), MHC class II, or the T cell receptor α or β chains (Mombaerts et al. 1993). Although none of these mice exhibit disease identical to human IBD (ulcerative colitis or Crohn's disease), many characteristic features are present including crypt abscesses, mucosal ulcerations and granulomas. Absence

of these features in γ_c-deficient mice could reflect a different etiology of IBD; however, further analysis is required, particularly with older mice. Interestingly, large numbers of *Helicobacter hepaticus* were observed in the crypts and lumens of the cecum and colon of $\gamma_c^{-/Y}$ males (Cao et al. 1995). While these findings are consistent with speculation that an immune response to gut bacterial flora (either antigen specific or mitogen driven) may contribute to the development of IBD in gene-targeted (Strober & Ehrhardt 1993, MacDonald 1994) and SCID (Russell et al. 1995) mice, a causal relationship between *H. hepaticus* and the inflammatory changes in the $\gamma_c^{-/Y}$ mice has not been established.

PHENOTYPIC ANALYSIS OF T AND B CELLS IN $\gamma_c^{-/Y}$ MICE

Although thymocytes were greatly diminished in total numbers, flow cytometric analysis of thymocytes revealed that $CD4^-CD8^-$ double negative (DN), $CD4^+CD8^-$ and $CD4^-CD8^+$ single positive (SP), and $CD4^+CD8^+$ double positive (DP) cells were all represented (Cao et al. 1995). The $\gamma_c^{-/Y}$ thymocytes appeared to include a relatively high proportion of mature cells, with a relative increase in the number of cells bright for TCRβ, CD3ϵ, and CD5, and a corresponding decrease in the number of cells expressing high levels of HSA. Interestingly, there was a significant increase in the $CD4^+CD8^-$:$CD4^-CD8^+$ ratio in the thymus. Although a role for specific cytokines and their receptors has not been established in positive selection, the increased percentage of $CD4^+$ thymocytes suggests this possibility. Splenocytes also exhibited an increase in the $CD4^+CD8^-$:$CD4^-CD8^+$ ratio (Cao et al. 1995), consistent with the skewing observed in the thymus. These appeared to be conventional $CD4^+$ T cells and not the population of class I MHC educated $CD4^+$ T cells (Bendelac et al. 1994, Coles & Raulet 1994, Lantz & Bendelac 1994) since few cells were TCR Vβ8 positive and they did not stain with antibody to NK1.1 (Cao et al. 1995). In fact, no NK1.1$^+$ cells were identified, consistent with the lack of natural killer cytolytic activity in $\gamma_c^{-/Y}$ mice (Cao et al. 1995). In contrast, granulocytes (Gr1$^+$ cells) and monocytes/macrophages (Mac1$^+$ cells) were increased.

Analysis of B cells in both spleen and bone marrow by staining for B220 and μ revealed a significant decrease in the number of conventional B cells. There was also a decrease in the number of B220$^+$ CD43$^-$ cells in the bone marrow of γ_c-deficient mice (unpublished data; DiSanto et al. 1995), consistent with a requirement for IL-7R in B cell development (Peschon et al. 1994). In contrast, "self replenishing" peritoneal B-1 cells (Kantor & Herzenberg 1993) were less affected. Both CD5$^+$ and CD5$^-$ B-1 cells were identified in γ_c-deficient mice. The presence of these cells may explain the detectable levels of serum IgM expressed in the $\gamma_c^{-/Y}$ mice; the other classses of immunoglobulin were greatly diminished (Cao et al. 1995). This finding was expected for IgG1 and IgE, given that in the absence of γ_c, IL-4 signaling should be defective, and is also consistent with the phenotype of IL-4$^{-/-}$ mice (Kuhn et al. 1991).

As mentioned above, humans with XSCID generally lack T cells, so it was surprising that significant numbers of T cells were found in $\gamma_c^{-/Y}$ mice (Cao et al. 1995). The T cells appeared to be strictly $\alpha\beta$ TCR$^+$ as $\gamma\delta$ T cells were not identified in the thymus or spleen. Moreover, examination of the skin revealed that although Langerhans cells were normal in number and distribution, $\gamma\delta$ TCR$^+$ dendritic epidermal T cells were absent. iIELs were also absent, and specific staining for $\gamma\delta$ TCR$^+$ cells in the intestine was negative. Thus, it appears that $\gamma_c^{-/Y}$ mice exhibit a total absence of all populations of $\gamma\delta$ T cells, whereas at least a subset of $\alpha\beta$ T cells are able to mature and populate the peripheral lymphoid tissue (Cao et al. 1995). The lack of $\gamma\delta$ T cells could be responsible in part for the relative paucity of $\alpha\beta$ TCR$^+$ thymocytes and T cells in $\gamma_c^{-/Y}$ mice as previous studies have suggested that $\gamma\delta$ TCR$^+$ thymocytes can contribute to the development of $\alpha\beta$ T cells (Shores et al. 1990, Ferrick et al. 1990, Iwashima et al. 1991).

The presence of T cells in the spleen but almost complete absence of lymph nodes in $\gamma_c^{-/Y}$ mice was unexpected. One possible explanation for these findings is that γ_c-dependent cytokine signals might participate in T cell homing to lymph nodes. Alternatively, in the absence of appropriately activated lymphocytes (and the factors they secrete), endothelial cells may not be induced to a state capable of supporting lymphocyte homing. While these remain intriguing possibilities, additional studies are required to clarify the basis for the decreased lymph node mass.

One of the most interesting findings in $\gamma_c^{-/Y}$ mice was the increase in numbers of CD4$^+$ T cells, particularly with advancing age. This is unlikely to be a result of expansion as peripheral T cells from these mice failed to proliferate in culture (Cao et al. 1995). The marked accumulation of T cells in $\gamma_c^{-/Y}$ mice with advancing age is also consistent with the proposed role of γ_c-mediated signals in peripheral negative selection. Whereas T cells stimulated through the TCR in the absence of co-stimulation usually enter a state of anergy, it has been shown that induction of anergy could be prevented if concomitant signals were delivered through γ_c (Boussiotis et al 1994). Results from another study suggest that IL-2 is important for the induction of cell death. High doses of antigen in combination with IL-2 have been shown to induce cell death both in antigen-specific cell lines and in a transgenic model of EAE (Critchfield et al. 1994). Hence, it is possible that the T cells that accumulate in $\gamma_c^{-/Y}$ mice represent "anergized" T cells with potentially autoreactive TCR specificities.

COMPARISON OF T- AND B-CELL ABNORMALITIES IN HUMANS WITH XSCID AND $\gamma_c^{-/Y}$ MICE

As noted above, in human XSCID, T cells are absent or profoundly diminished in numbers. In contrast, in $\gamma_c^{-/Y}$ mice, though diminished, T cells are present and with

time can increase to essentially normal numbers in the spleen (Cao et al. 1995). Although peripheral T cells from $\gamma_c^{-/Y}$ mice did not respond to stimulation with either concanavalin A or to PMA + IL-4, stimuli strictly dependent on γ_c, there was modest proliferation to anti-CD3 + anti-CD28 and to PMA + ionomycin (Cao et al. 1995). Splenocyte proliferation was approximately 10 to 20% of wild-type control levels with PMA + ionomycin and approximately 5% of control levels with anti-CD3 + anti-CD28 (Cao et al. 1995). However, quite unexpectedly, thymocytes from γ_c-deficient mice proliferated as well as control thymocytes in response to stimulation with anti-CD3 + anti-CD28 (Figure 2; Cao et al. 1995).

These findings were surprising for at least two reasons. First, they indicated that T-cell development could proceed in the absence of γ_c in mice. Second, they revealed that T cells, but more strikingly thymocytes, can be stimulated to proliferate independently of γ_c-mediated signals. Both of these findings could be explained by the actions of cytokine(s) whose receptors do not contain of γ_c. Although it is unclear which cytokine(s) might be responsible, it may be relevant that IL-7R$^{-/-}$ mice exhibit a more severe phenotype (Peschon et al. 1994) than do $\gamma_c^{-/Y}$ mice (Cao et al. 1995). This finding initially seems remarkable given that the absence of γ_c should inactivate IL-2, IL-4, IL-7, IL-9, and IL-15 signaling. Thus, one might assume that IL-7R$^{-/-}$ mice would respond to all cytokines except IL-7. However, this may not in fact be the case. Loss of IL-7R would also inactivate signaling in response to another cytokine, thymic stromal derived lymphopoietin (TSLP) (Peschon et al. 1994). The TSLP receptor contains IL-7R but it has not been determined whether it contains γ_c. Thus, we speculate that TSLP action contributes to T-cell development in the $\gamma_c^{-/Y}$ mice (Cao et al. 1995). Since no human homologue of TSLP has been identified, it is conceivable that humans with XSCID lack this cytokine to compensate for the loss of IL-7 action and therefore exhibit more severe defects than do $\gamma_c^{-/Y}$ mice.

Regarding B-cell development, it is notable that IL-7R$^{-/-}$ mice, IL-7R$^{-/-}$ mice and mice treated with anti-IL-7 antibodies both exhibit a block in B-cell development (von Freeden-Jeffry et al. 1995, Peschon et al. 1994, Grabstein et al. 1993). Thus, in $\gamma_c^{-/Y}$ mice, it seems likely that the block in development of conventional B cells (Cao et al. 1995) results from inactivation of IL-7 signaling, consistent with the action of IL-7 as a pre-B-cell growth factor. These findings also support the contention that B-1 peritoneal B cells, whose development is less affected in $\gamma_c^{-/Y}$ mice, are derived from a separate lineage from conventional B cells (Kantor & Herzenberg 1993). It is striking that in human XSCID, B cells are normal or even increased in number, albeit nonfunctional. This observation suggests either that IL-7 is a major pre-B cell growth factor in mice, but not in humans, or that alternative γ_c-independent pathway(s) exist to support B-cell development in humans. Although this hypothesis is attractive, it should be emphasized that other explanations could exist to explain the observed differences between XSCID patients and $\gamma_c^{-/Y}$ mice. For example, the degree to which maternal factors (eg., cytokines) influence fetal development may not be identical in both species.

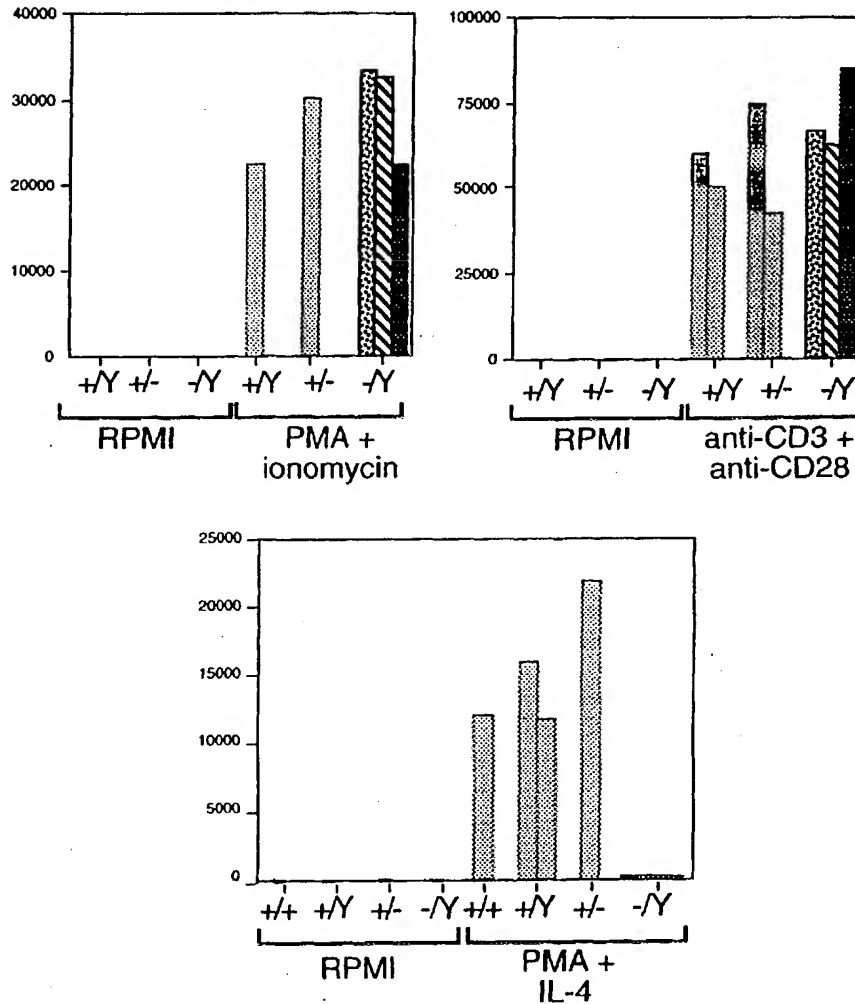# THYMOCYTES FROM γ_C-KNOCKOUT MICE RESPOND TO γ_C-INDEPENDENT BUT NOT γ_C-DEPENDENT STIMULI

Figure 2. Thymocytes from $\gamma_c^{-/Y}$ mice respond to PMA + ionomycin and yo anti-CD3 + anti-CD28 (γ_c-independent stimuli), but not to PMA + IL-4 (a γ_c-dependent stimulus).

COMPARISON OF IMMUNOLOGICAL DEFECTS ASSOCIATED WITH MUTATION OF THE GENES ENCODING IL-2, IL-2Rα, IL-2Rβ, AND γ_c

The first component of the IL-2/IL-2 receptor system to be analyzed in a murine system was IL-2 (Schorle et al. 1991). These mice develop normally during the first 3–4 weeks of life and exhibit normal thymocyte and peripheral T-cell subset composition. Some mice subsequently develop splenomegaly, lymphadenopathy, and severe anemia and die between 4 and 9 weeks of age (Sadlack et al. 1993). Of those that survive, 100% develop a form of IBD that resembles ulcerative colitis in humans (Sadlack et al. 1993). This syndrome is characterized by chronic diarrhea, intestinal bleeding, and rectal prolapse, as well as by the microscopic detection of crypt abscesses and mucosal ulcerations. Interestingly, the IL-2$^{-/-}$ mice not only exhibit splenomegaly and lymphadenopathy, but have a high number of activated T and B cells in the colonic mucosa and drastically elevated IgG1 and autoantibody production.

Mice lacking IL-2Rβ expression would be expected to manifest a simultaneous loss of IL-2 and IL-15 signaling. The IL-15 receptor is analogous to the IL-2 receptor in that they both share IL-2Rβ and γ_c (Grabstein et al. 1994, Bamford et al. 1994, Giri et al. 1994). They differ only in the expression of different α chains, each of which has a relatively short cytoplasmic domain. So far, it appears that IL-2 and IL-15 induce identical signals when cells can respond to both cytokines (Grabstein et al. 1994, Bamford et al. 1994, Giri et al. 1994, Lin et al. 1995). Thus, if IL-2Rβ were restricted solely to IL-2 and IL-15 signaling, the phenotype might be expected to be quite similar to that found in IL-2 deficient mice. In IL-2Rβ$^{-/-}$ mice, thymic development was normal between 1 and 3 weeks of age, suggesting that IL-2Rβ is not required for T-cell development in the thymus (Suzuki et al. 1995). Interestingly, however, by 6 weeks of age, absolute numbers of thymocytes were only approximately 15% of normal levels. Since there was an increase in single positive and decrease in double positive thymocytes, this could potentially reflect general stress-induced changes. By 3 weeks of age, homozygous mice exhibited splenomegaly and lymphadenopathy, with large lymphoblastoid T cells, albeit in a normal CD4$^+$CD8$^-$ to CD4$^-$CD8$^+$ ratio. The activated CD4 cells collectively synthesized both T$_h$1 (γ-interferon) and T$_h$2 (IL-4) cytokines and stimulated synthesis of high levels of serum immunoglobulin by B cells, including autoantibodies. These included anti-erythrocyte antibodies that likely mediate the autoimmune hemolytic anemia found in IL-2Rβ$^{-/-}$ mice. Although the T cells were activated, there was no response to specific antigen nor to polyclonal activators. B- to T-cell ratios in lymph nodes were normal at three weeks of age, but by 8 weeks of age, B cells had essentially disappeared; this could be prevented by blocking the increase in CD4$^+$ T cells by treatment with anti-CD4 antibodies. Thus, in the absence of IL-2Rβ, there is a disruption of normal lymphoid homeostasis.

Mice lacking IL-2Rα expression initially have normal development of T and B

cells, but as adults, they exhibit marked lymphoid expansion with polyclonal T- and B-cell activation (Willerford et al. 1995). With time, autoimmune disorders, including inflammatory bowel disease and hemolytic anemia develop. Mice lacking IL-2Rα expression are most similar to the IL-2 knockout mice, but nevertheless some differences exist (Willerford et al. 1995). Among the possible explanations for the differences would be the known ability of IL-2 to trigger certain responses via intermediate affinity IL-2 receptors (Siegel et al. 1987), which contain IL-2Rβ and γ_c, but not IL-2Rα.

In the IL-2, IL-2Rα, IL-2Rβ, and γ_c knockout mice, it is interesting that there is a transition point at approximately 3 weeks of age. In γ_c knockout mice, this represents the point at which the spleen enlarges and CD4$^+$ T-cell expansion occurs, whereas for the IL-2Rβ deficient mice, this represents the point at which thymic involution occurs, and for the IL-2 knockout mice, it is at approximately 4 weeks of life that splenomegaly, lymphadenopathy, and severe anemia develop.

The fact that the phenotypes of IL-2$^{-/-}$, IL-2Rα$^{-/-}$, IL-2Rβ$^{-/-}$, and $\gamma_c^{-/Y}$ mice are all different emphasizes the complexity of cytokine–cytokine receptor systems. The principles of "cytokine pleiotropy" and "cytokine redundancy" have been reviewed in detail (Paul 1989, Leonard 1994). In addition, however, one also needs to consider the principles of "cytokine receptor subunit pleiotropy", and "cytokine receptor subunit redundancy". Cytokine pleiotropy refers to multiple actions by a single cytokine, often on multiple cell types, whereas cytokine redundancy refers to the settings where several cytokines exert overlapping actions, making it difficult to know *a priori* how severe the loss of any single cytokine will be. In effect, if a biological "function" is important, it may be subserved by more than one cytokine. Cytokine receptor pleiotropy refers to the rather well established principle that individual receptor components may act in multiple different cytokine systems. Although this has been discussed above for β_c, γ_c, and gp130, there are an ever increasing number of receptor molecules that have been recognized to be shared by multiple cytokines (see Table II). Finally, there is the interesting phenomenon of cytokine receptor redundancy, a principle perhaps best illustrated in the murine IL-3 system, wherein IL-3 signaling requires a functional IL-3Rα and either the common β chain (β_c) or an IL-3 receptor specific β chain that shares 91% amino acid identity to β_c (Miyajima et al 1992). IL-3Rβ cannot work with IL-5 or GM-CSF and does not exist in humans. For the mouse, however, IL-3Rβ can fully replace β_c in mediating IL-3 action. A more confusing example appears to exist for IL-4. In this setting, it is clear that IL-4R is required for IL-4 actions, but depending on the cell type, either γ_c or an alternate additional chain (operationally denoted γ' and hypothesized to potentially be the IL-13R [Lin et al. 1995]) can transduce IL-4 signals. It appears that T-cell responses to IL-4 are strictly dependent on γ_c, whereas IL-4 responses on certain other cells can be mediated in the absence of γ_c and presumably require the alternate chain. It is also possible that the specific signals induced by IL-4 may depend in part on the type of receptor expressed on a given cell. If so, then

TABLE II

Cytokine receptor pleiotropy: involvement of receptor subunits as components of multiple cytokine receptors

Shared Chain	Cytokines whose receptors contain the shared chain
γ_c	IL-2, IL-4, IL-7, IL-9, IL-15
gp130	IL-6, leukemia inhibitory factor (LIF), oncostatin M (OSM), ciliary neurotrophic factor (CNTF), IL-11, cardiotrophin-1
β_c	IL-3, IL-5, and GM-CSF
LIF receptor	LIF, OSM, and CNTF
IL-2Rβ	IL-2, IL-15
IL-4R	IL-4, IL-13
IL-7R	IL-7, TSLP

in this instance the principle of cytokine receptor subunit redundancy would not truly establish a redundant situation but rather would explain the basis for pleiotropic actions by IL-4. In any case, the complicated network of cytokines and cytokine receptor chains makes it difficult to predict with certainty the outcome of inactivation of any individual cytokine or receptor chain. In fact, the creation of mice defective in individual chains has allowed a greater understanding of the roles that various proteins may play.

SUMMARY

To examine the role of γ_c in lymphoid development, we have analyzed mice in which the γ_c gene was specifically inactivated by homologous recombination. These mice also serve as an animal model of human X-linked severe combined immunodeficiency (XSCID). Interestingly, γ_c knockout mice exhibited a somewhat different phenotype than humans with XSCID. Absolute T-cell numbers are greatly diminished in young $\gamma_c^{-/Y}$ mice, but accumulate with age. $\gamma\delta$ T cells and NK cells are absent in $\gamma_c^{-/Y}$ mice and conventional B cells are greatly diminished, yet substantial numbers of peritoneal B-1 cells are present. Since humans with XSCID have essentially no mature T cells, it is especially striking that T cells are readily apparent in $\gamma_c^{-/Y}$ mice. This observation indicates that in these mice, the γ_c-dependent block in T cell development is less severe than it is in humans. It is possible but unproven that thymic stromal derived lymphopoietin, TSLP, contributes to thymocyte development in these mice. Since B-cell numbers are normal in humans with XSCID, it is also striking that $\gamma_c^{-/Y}$ mice paradoxically exhibit greatly diminished numbers of B cells. This likely indicates that IL-7 signaling plays a critical role in pre-B cell maturation in mice but is less important in humans. Thus, the abnormalities observed in $\gamma_c^{-/Y}$ mice have provided clues to assist in dissecting the

role of cytokines and their receptors in lymphoid development and have also identified interesting differences in the regulation of this process in mice and humans.

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Defects in heart and lung development in compound heterozygotes for two different targeted mutations at the N-*myc* locus

Cecilia B. Moens[1,3], Brian R. Stanton[2], Luis F. Parada[2] and Janet Rossant[1,3,*]

[1]Division of Molecular and Developmental Biology, Samuel Lunenfeld Research Institute, 600 University Ave, Toronto, Ontario, M5G 1X5, Canada
[2]Molecular Embryology Section, ABL-Basic Research Program, National Cancer institute - Frederick Cancer Research and Development Center, Frederick, Maryland 21702-1201, USA
[3]Department of Molecular and Medical Genetics, University of Toronto

*Author for correspondence

SUMMARY

Two types of mutant allele, one leaky and one null, have been generated by gene targeting at the N-*myc* locus in embryonic stem cells and the phenotypes of mice homozygous for these mutations have been described. These mutations have shown that N-*myc* has a number of functions during development, including a role in branching morphogenesis in the lung, which manifests itself at birth in mice homozygous for the leaky allele, and roles in the development of the mesonephric tubules, the neuroepithelium, the sensory ganglia, the gut and the heart, which become evident at midgestation in embryos homozygous for the null allele. In an attempt to define roles for N-*myc* at other stages of development, we have combined the two types of N-*myc* mutant allele in a compound heterozygote that as a result contains approximately 15% of normal levels of N-Myc protein. Compound heterozygotes died during gestation at a time

intermediate to the times of death of embryos homozygous for either mutation individually, and their death appeared to result from cardiac failure stemming from hypoplasia of the compact subepicardial layer of the myocardium. Investigation of the expression pattern of N-*myc* and various markers of differentiation in wild-type and compound heterozygote mutant hearts has suggested that N-*myc* may function in maintaining the proliferation and/or preventing the differentiation of compact layer myocytes. This study illustrates the importance of generating different mutations at a given locus to elucidate fully the function of a particular gene during development.

Key words: N-myc, heart development, targeted mutagenesis, mouse mutant

INTRODUCTION

N-*myc* is a member of the *myc* family of proto-oncogenes, which includes N-*myc*, c-*myc*, and L-*myc*. Myc proteins are site-specific DNA-binding proteins (Blackwell et al., 1990; Prendergast and Ziff, 1991; Alex et al., 1992), belonging to the basic-helix-loop-helix class of transcription factors, which includes genes that control cell fate determination in such diverse processes as myogenesis, neurogenesis and sex determination (reviewed in Garrell and Campuzano, 1991).

Deregulated expression of *myc* genes has been implicated in the genesis or progression of a number of naturally occurring tumours, in the transformation of cells in culture and in the formation of tumours in transgenic mice (reviewed in DePinho et al., 1991). In general, the sites of expression of a given *myc* gene in vivo reflect the types of tumours associated with its elevated expression. Thus N-*myc* is expressed predominantly in the embryo where it is restricted to undifferentiated subsets of cells in the central and peripheral nervous system, lung, kidney and eye

(Mugrauer et al., 1988; Hirning et al., 1991; Zimmerman et al., 1986) and overexpression of N-*myc* has been associated with tumours of embryonic origin such as neuroblastoma (Kohl et al., 1983; Schwab et al., 1983), small-cell lung cancer (Nau et al., 1986; Wong et al., 1986), Wilm's tumour (Nisen et al., 1986) and retinoblastoma (Lee et al., 1984). N-*myc* is also expressed in the skin (Mugrauer et al., 1988), in the epithelial layer of the intestine (Hirning et al., 1991) and, earlier in development, in the heart, sclerotome and visceral arches (Katoh et al., 1991).

The functioning of a Myc protein in vivo should depend not only on its own level of expression, but also on the levels of Max, a protein which, like the Myc proteins, possesses a basic-helix loop helix-leucine zipper (bHLH-LZ) domain (Blackwood and Eisenman, 1991), and which associates with N-Myc, L-Myc and c-Myc proteins in vivo (Blackwood et al., 1992; Wenzel et al., 1991; Mukherjee et al., 1992). Max is required for specific DNA binding by Myc proteins (Blackwood and Eisenman, 1991; Prendergast and Ziff, 1991; Kato et al., 1992; Barrett et al., 1992), and has

been shown to be required for transcriptional activation (Amati et al., 1992) and transformation (Amati et al., 1993) by c-*myc*. Unlike Myc proteins, Max is able to form homodimers in vitro and thereby to bind the *myc*-binding site (Prendergast and Ziff, 1991; Kato et al., 1992). However Max does not transactivate downstream genes on its own (Amati et al., 1992; Kretzner et al., 1992) because it lacks a transactivation domain (Kato et al., 1992) which Myc proteins possess (Kato et al., 1990). Both transformation of fibroblasts by c-*myc* and N-*myc* (Mukherjee et al., 1992; Makela et al., 1992; Prendergast et al., 1992) and transcriptional transactivation by c-*myc* (Amati et al., 1992; Kretzner et al., 1992) have been shown to be enhanced by low levels of Max and inhibited by excess Max, suggesting that Myc function is indeed influenced by levels of Max. Recently, bHLH-LZ proteins have been isolated which also bind the Myc recognition site and which suppress transcription as heterodimers with Max (Ayer et al., 1993; Zervos et al., 1993). One of these, Mad (Ayer et al., 1993), has been shown to compete with Myc for Max in vitro and in transfected cells. Thus high levels of expression of Max dimerization partners may indirectly affect Myc function in vivo by competing for available Max protein and for DNA-recognition sites. Finally, Myc function in vivo may be affected by the levels of other Myc proteins (Mukherjee et al., 1992; Resar et al., 1993).

In an effort to understand the function of *myc* genes in embryogenesis, leaky and null mutations were made in N-*myc* in embryonic stem cells by homologous recombination (Charron et al., 1990; Stanton et al., 1990; Sawai et al., 1991; Moens et al., 1992). These mutations have allowed a description of the function of N-*myc* at different stages of development. Mice homozygous for the null mutations die at midgestation (Stanton et al., 1992; Charron et al., 1992) while mice homozygous for the leaky mutation survive until birth, when they die due to a defect in lung branching morphogenesis (Moens et al., 1992). The latter phenotype is consistent with the normal expression of N-*myc* in the developing lung epithelium, and has led us to postulate that N-*myc* plays a role in the response of the lung epithelium to the signals from the lung mesenchyme that induce epithelial branching.

There have been two detailed reports of the phenotype of mice carrying null mutations in N-*myc* (Stanton et al., 1992; Charron et al., 1992). In the homozygous condition, these mutations cause embryonic death at around 11.5 days p.c. and cause hypoplasia in a number of components of the embryo, different aspects of which have been emphasized by the two groups. The developing genitourinary system shows a reduced number of mesonephric tubules in mutant embryos, which is consistent with N-*myc*'s normal expression in the newly induced epithelium of the meso- and metanephric tubules (Katoh et al., 1991; Mugrauer et al., 1988). The genital ridge is also hypoplastic. Stanton et al. (1992) also describe a defect in the development of the stomach and intestine, consistent with N-*myc*'s expression in the epithelium of these tissues (Hirning et al., 1991). Homozygotes also have reduced cranial and spinal ganglia and a thin neuroepithelium in the telencephalon; both of these are sites of N-*myc* expression in the embryo. Finally, homozygotes have a defect in heart development in which

the heart appears to cease development at 9.5 days p.c. (Charron et al., 1992) and as a result is poorly compartmentalized and appears not to have undergone the normal epithelial-to-mesenchymal transitions that result in the formation of the septa and valves. This is again consistent with a previous description of N-*myc* expression in the myocardium of the 9.5 day embryo (Katoh et al., 1991).

By combining leaky and null alleles of N-*myc* in a compound heterozygote, we hoped to investigate N-*myc* function in the mouse embryo at a stage intermediate to those identified by the null and leaky alleles individually. We find that compound heterozygotes indeed survive longer than null homozygotes, but that they die before birth. The mutant phenotype includes a more severe defect in branching morphogenesis of the lung, as well as a cardiac myocyte hypoplasia in the compact subepicardial layer of the ventricular myocardium. We show that, consistent with this phenotype, N-*myc* expression in the developing myocardium is restricted to the compact layer. A role for *myc* genes in the control of myocyte differentiation in the heart is discussed.

MATERIALS AND METHODS

PCR genotyping of embryos

Yolk sacs from dissected embryos were washed several times in fresh PBS and placed in 100-200 µl of proteinase K buffer with non-ionic detergents (50 mM KCl, 10 mM Tris pH 8.3, 2.0 mM $MgCl_2$, 0.1 mg/ml gelatin, 0.45% Nonidet P-40, 0.45% Tween-20). 10 µg of proK was added to each tube and samples were incubated at 55°C overnight. 10 µl of the mixture was placed in a PCR tube with 30 µl sterile water and heated to 94°C for 10 minutes before cooling to 62°C. 0.1 µg of each primer, 200 µM dNTPs, 10× reaction buffer and Taq polymerase (Promega) were added and PCR was run with an annealing temperature of 62°C (1 minute), an extension temperature of 72°C (2 minutes), and a denaturing temperature of 94°C (1 minute). Each consecutive cycle was extended by 2 seconds. The oligonucleotide primers used for the PCR detection of 9a, BRP and wild-type alleles of N-*myc* are shown in Fig. 1. Their sequences are as follows: primer a (N-*myc* intron 1): 5′-GGTAGTCGCGCTAGT AAGAG-3′; primer b (N-*myc* exon 2): 5′-GGCGTGGGCA GCAGCTCAAAC-3′; primer c (N-*myc* intron 2): 5′-CCGAGCATCTGTCGCCAAGTC-3′; primer d (neo): 5′-GACCGCTATCAGGACATAGCG-3′.

Western blotting

An anti-human N-*myc* monoclonal antibody, NCMII 100 (Ikegaki et al., 1986), was generously provided by Dr R. Kennett. 11.5 days p.c. embryos from 9a/+×BRP/+ crosses were dissected and homogenized in several volumes of 1× sample buffer without bromophenol blue (60 mM Tris, pH 6.8, 2% SDS, 0.1 M DTT, 0.32% pharmalyte 3-10 (Pharmacia)). Samples were boiled for 5 minutes and chromosomal DNA was sheared by repeated passage through a 26-gauge needle before freezing at −20°C for future use. Yolk sacs were washed several times in PBS and were prepared for and typed by PCR as described above. After typing, extracts of embryos of each genotype were pooled and the concentration of protein in each sample was determined by the Bradford assay (protein assay reagent from Biorad). Approximately 20 µg of protein from each genotype in 2× loading buffer (0.1 M Tris pH 6.8, 20% glycerol, 4% SDS, 0.2% bromophenol blue, 0.2 M DTT) was run in a 10% SDS-PAGE. Protein was transferred onto PVDF membrane (Immobilon-P, Millipore). Application of primary antibody and fixation of the primary antibody to the blot with 0.2%

glutaraldehyde was performed as described (Ikegaki and Kennett, 1989). Horseradish peroxidase-conjugated goat anti-mouse secondary antibody and reagents for the chemiluminescent detection of HRP activity were obtained in the ECL western blotting kit (Amersham) and were used as suggested by the manufacturer.

Histology

Dissected embryos were fixed in 10% buffered formalin overnight at room temperature, then were dehydrated and cleared by soaking sequentially in 70%, 80%, 90% and 95% ethanol, 1:1 ethanol:xylene, xylene, 1:1 xylene:paraffin wax, (TissuePrep 2, Fisher Scientific) each for 1 hour. Embryos were then oriented and embedded in paraffin wax. Mutant embryos and control littermates were oriented either for sagittal or frontal sections. 5 μm sections were placed on slides, which were dried overnight at 37°C. Slides were dewaxed, rehydrated, and stained with hematoxylin and eosin. To analyse mutant phenoypes, sections throughout wild-type and mutant embryos were carefully examined in order to find those sections that passed through corresponding regions of the different embryos. Mutant embryos were always compared to non-mutant littermates rather than to non-mutant embryos from other litters, in order to rule out differences due to age.

RNA in situ hybridizations

Probes for in situs were as follows. N-*myc*: a 541 bp *Pst*1-*Sca*1 fragment of the N-*myc* genomic clone N7.7 (DePinho et al., 1986), including largely 3'-untranslated sequence; c-*myc*: a 351 bp *Hae*III fragment including the untranslated first exon (Bossone et al., 1992); *flk-1*: an 800 bp fragment spanning the transmembrane domain and part of the extracellular ligand-binding domain (Yamaguchi et al., 1993) and α-cardiac actin, a 130 bp *Bam*HI fragment including the first untranslated exon of the mouse α-cardiac actin gene (Sassoon et al., 1988). In situ hybridization was carried out essentially as described (Frohman et al., 1990) with the following modifications: in vitro-transcribed, α-^{35}S-labeled RNA probes were used at 5×10^4 disintegrations/minute per 1 μl of hybridization solution for the α-cardiac actin probe, and at 1×10^5 disintegrations/minute per 1 μl of hybridization solution for c-*myc*, N-*myc* and *flk-1* probes. Slides were hybridized at 53°C overnight, and were washed in the presence of 0.1% β-mercaptoethanol as a reducing agent. High stringency washes were in 50% formamide, 0.1% β-mercaptoethanol, and 1× SSC at 65°C. Final rinses in 2× and 0.1× SSC were at 65°C for 15 minutes. Slides were exposed for either 6 days (for α-cardiac actin probe) or for 14 days (for N-*myc*, *flk-1* and c-*myc* probes). After developing and photographing with dark-field illumination, slides were stained with hematoxylin and eosin and were rephotographed with bright-field illumination.

RESULTS

Time of death of N-*myc* compound heterozygotes

The leaky mutation that was generated in N-*myc* (Moens et al., 1992) was termed N-*myc*^{9a} and the null allele of N-*myc* used in this study was named N-*myc*^{BRP} (Stanton et al., 1992). Both the N-*myc*^{9a} mutation and the N-*myc*^{BRP} mutation are lethal in the homozygous condition. Therefore, N-*myc*$^{9a/BRP}$ embryos were generated by crossing N-*myc*$^{9a/+}$ females to N-*myc*$^{BRP/+}$ males, with the expected ratio of embryos being 1 N-*myc*$^{+/+}$: 1 N-*myc*$^{9a/+}$: 1 N-*myc*$^{BRP/+}$: 1 N-*myc*$^{9a/BRP}$. The reciprocal cross was also performed: no differences in mutant phenotypes were observed.

In order to expedite the genotypic analysis of offspring from this cross, a PCR strategy was designed that used four oligonucleotide primers to distinguish 9a, BRP, and wild-type alleles of N-*myc* (Fig. 1). 161 live offspring from N-*myc*$^{9a/+}$×N-*myc*$^{BRP/+}$ crosses were typed by PCR between 1 day and 3 weeks after birth. Among these pups, 59 were N-*myc*$^{+/+}$, 53 were N-*myc*$^{9a/+}$ and 49 were N-*myc*$^{BRP/+}$. No live animals were N-*myc*$^{9a/BRP}$. No pups were observed to die postnatally as occurred in the case of N-*myc*$^{9a/9a}$ newborns, suggesting that the N-*myc*$^{9a/BRP}$ phenotype was indeed more severe than the N-*myc*$^{9a/9a}$ phenotype.

We dissected embryos from N-*myc*$^{9a/+}$×N-*myc*$^{BRP/+}$ crosses at various stages of gestation in order to determine the time during gestation when N-*myc*$^{9a/BRP}$ embryos died. Between 8.5 and 11.5 days p.c., N-*myc*$^{9a/BRP}$ embryos were phenotypically normal and constituted approximately 25% of the total number of embryos, by PCR analysis. Of 268 embryos dissected between 8.5 and 11.5 days p.c., 61 (23%) were N-*myc*$^{9a/BRP}$ embryos. This is clearly different from the situation in N-*myc*$^{BRP/BRP}$ embryos, which were all found to be dead by 11.5 days p.c. (Stanton et al., 1992). The observed differences in phenotype among the various mutants were unlikely to be due to different genetic background effects, since all mutant phenotypes were analysed in outbred mice.

Occasionally, N-*myc*$^{9a/BRP}$ embryos at 11.5 days were slightly smaller than their littermates but were otherwise indistinguishable from their wild-type or single heterozygote littermates. However, by 12.5 days, most N-*myc*$^{9a/BRP}$ embryos were distinguishable from their littermates either because they were dead and necrotic, or because they were alive but had a characteristic edema of the body wall in the neck area (Fig. 2). Subsequent histological analysis identified this swelling as likely being the result of extravasion of fluid from the vascular compartment into the connective tissue, since the jugular veins are dilated in these embryos (Fig. 7g,h). Of 230 embryos dissected at 12.5 days p.c., 42 (18%) were N-*myc*$^{9a/BRP}$, but among these, 12 were dead and necrotic, 23 had the characteristic edema described above, two were smaller than normal and five were phenotypically normal. After 12.5 days p.c., live N-*myc*$^{9a/BRP}$ embryos were progressively less frequent and all were phenotypically abnormal. N-*myc*$^{9a/BRP}$ embryos that survived until 14.5 days p.c. were always smaller than normal and had a large edema in the neck area. Live N-*myc*$^{9a/BRP}$ embryos were not observed after 14.5 days p.c. Coincident with the loss of N-*myc*$^{9a/BRP}$ embryos there was an increase in the number of resorptions visible in the uterus. These resorptions were not typed, but presumably were derived from N-*myc*$^{9a/BRP}$ embryos, since the sum of the number of N-*myc*$^{9a/BRP}$ embryos and the number of resorptions added up to approximately 25% of the total number of embryos and resorptions at each stage of gestation. These data are shown graphically in Fig. 3.

N-Myc protein levels in mutant embryos

Although we have previously shown reduced N-*myc* mRNA levels in N-*myc*$^{9a/9a}$ embryos (Moens et al., 1992), N-Myc protein levels have not been determined in mice bearing either the leaky or the null N-*myc* alleles. In the present study, we have used western blot analysis to assess N-Myc protein levels in embryos with the various N-*myc* genotypes. Fig. 4 shows a western blot performed on
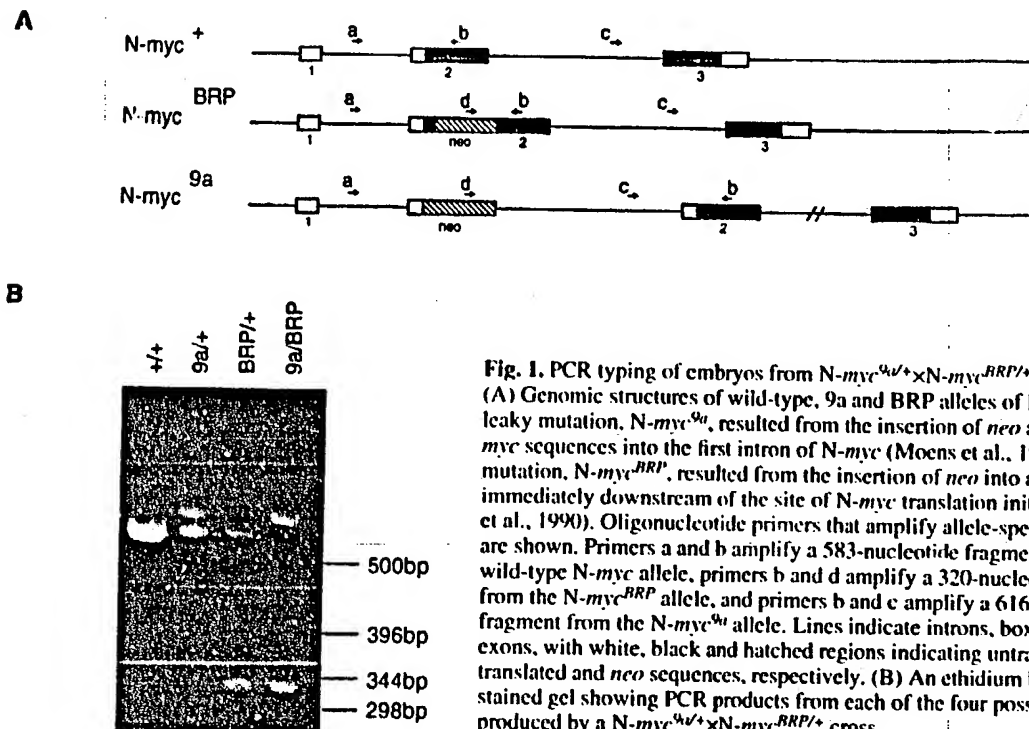
A

B

Fig. 1. PCR typing of embryos from N-*myc*⁹ᵃ/⁺×N-*myc*ᴮᴿᴾ/⁺ crosses.
(A) Genomic structures of wild-type, 9a and BRP alleles of N-*myc*. The leaky mutation, N-*myc*⁹ᵃ, resulted from the insertion of *neo* and flanking N-*myc* sequences into the first intron of N-*myc* (Moens et al., 1992). The null mutation, N-*myc*ᴮᴿᴾ, resulted from the insertion of *neo* into a Xho site immediately downstream of the site of N-*myc* translation initiation (Stanton et al., 1990). Oligonucleotide primers that amplify allele-specific fragments are shown. Primers a and b amplify a 583-nucleotide fragment from the wild-type N-*myc* allele, primers b and d amplify a 320-nucleotide fragment from the N-*myc*ᴮᴿᴾ allele, and primers b and c amplify a 616-nucleotide fragment from the N-*myc*⁹ᵃ allele. Lines indicate introns, boxes indicate exons, with white, black and hatched regions indicating untranslated, translated and *neo* sequences, respectively. (B) An ethidium bromide-stained gel showing PCR products from each of the four possible genotypes produced by a N-*myc*⁹ᵃ/⁺×N-*myc*ᴮᴿᴾ/⁺ cross.

extracts of whole 11.5 days p.c. embryos from a N-*myc*⁹ᵃ/⁺×N-*myc*ᴮᴿᴾ/⁺ cross, using a monoclonal antibody that was raised against human N-Myc protein (Ikegaki et al., 1986), but which also recognizes mouse N-Myc protein. N-Myc protein runs at approximately 65×10^3 M_r. This N-*myc* antibody also showed binding to a protein of approximately 100×10^3 M_r. The relative intensities of this cross-reacting band between lanes matched the relative intensities of a ubiquitously expressed protein phosphatase, *syp* (not shown), so this band was used as a loading control in scanning densitometry. The amount of N-Myc protein in N-*myc*⁹ᵃ/ᴮᴿᴾ embryos was considerably lower than in their wild-type or singly heterozygous littermates. While N-*myc*⁹ᵃ/⁹ᵃ embryos had approximately 25% of wild-type levels of N-Myc protein (not shown), N-*myc*⁹ᵃ/ᴮᴿᴾ embryos had approximately 15% of wild-type levels, as determined by scanning densitometry of this and several other, similar Western blots. This is consistent with the more severe phenotype of N-*myc*⁹ᵃ/ᴮᴿᴾ embryos. N-*myc*ᴮᴿᴾ/⁺ embryos had approximately 50% of wild-type levels of N-Myc protein, consistent with the N-*myc*ᴮᴿᴾ mutation being a null mutation.

Histological analysis of N-*myc*⁹ᵃ/ᴮᴿᴾ embryos

In order to determine more precisely the phenotype of N-*myc*⁹ᵃ/ᴮᴿᴾ embryos, histological analysis of hematoxylin and eosin-stained, sectioned embryos was performed at various stages of development. We examined a total of 42 N-*myc*⁹ᵃ/ᴮᴿᴾ embryos and 50 N-*myc*⁺/⁺ littermates in this manner (5 N-*myc*⁹ᵃ/ᴮᴿᴾ embryos at 14.5 days p.c., 28 at 12.5 days p.c., 3 at 11.5 days p.c., and 6 at 10.5 days p.c.). Younger embryos were analyzed without sectioning, after whole-mount RNA in situ hybridization with various

probes (data not shown). Occasionally, N-*myc*⁹ᵃ/⁺ embryos were used as controls since we have previously shown that the N-*myc*⁹ᵃ mutation has no phenotypic effect in the heterozygous condition. N-*myc*⁹ᵃ/ᴮᴿᴾ embryos not used for histology were used for N *Myc* protein analysis and RNA in situ analysis.

Before 12.5 days p.c., N-*myc*⁹ᵃ/ᴮᴿᴾ embryos were apparently normal and could not generally be distinguished from their littermates either by gross morphology or by histological examination of sectioned embryos. The first signs of lethality occurred at 12.5 days, when 12 out of 42 N-*myc*⁹ᵃ/ᴮᴿᴾ were clearly in the process of resorption. Aside from the edema in the neck area, described above, and their slightly smaller size, live N-*myc*⁹ᵃ/ᴮᴿᴾ embryos at 12.5 days were still not grossly abnormal. However careful examination of specific organ systems did reveal particular defects.

Lung development

N-*myc*⁹ᵃ/⁹ᵃ mice die at birth due to a defect in lung branching morphogenesis, consistent with the observation that N-*myc* is expressed in the lung epithelium at early stages of lung development (Moens et al., 1992). Specifically, at 12.5 days p.c., when wild-type lungs have tertiary and quaternary branches, N-*myc*⁹ᵃ/⁹ᵃ lungs had only the beginnings of tertiary branches. The further reduction in the amount of N-Myc protein in N-*myc*⁹ᵃ/ᴮᴿᴾ embryos led to an enhancement of this phenotype, as predicted. Branching morphogenesis in N-*myc*⁹ᵃ/ᴮᴿᴾ lungs was all but blocked (Fig. 5A,B), N-*myc*⁹ᵃ/ᴮᴿᴾ embryos having only the beginnings of secondary branches. Interestingly, the *pattern* of branching was normal, as can be observed by the presence of a rudimentary right postcaval lobe extending to the left
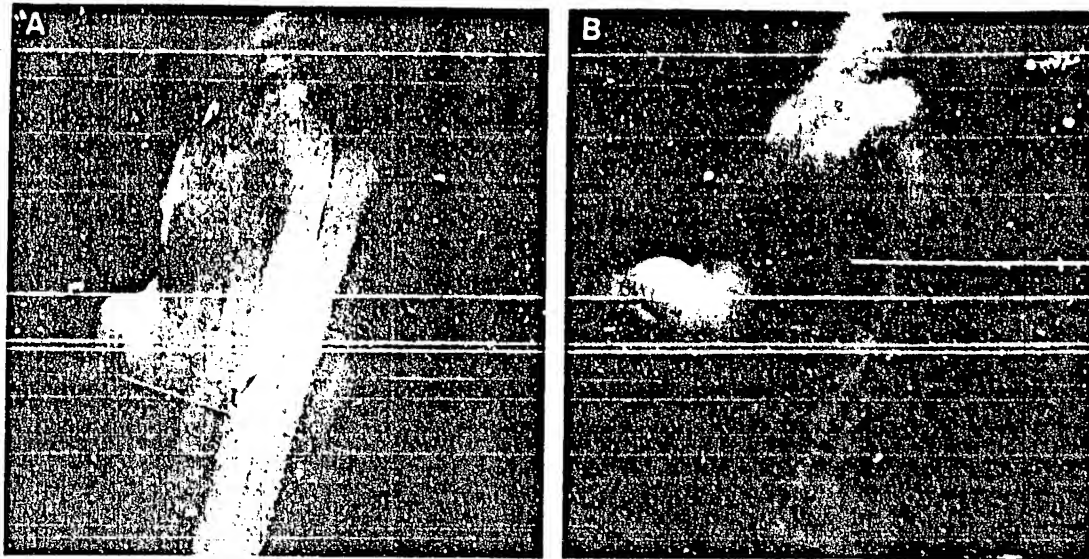
Fig. 2. N-*myc*$^{9a/BRP}$ embryos are edematous in the region of the neck. (A) Wild-type 12.5 days p.c. embryo, dorsal view. (B) N-*myc*$^{9a/BRP}$ embryo, showing swelling of the tissue and expanded, blood-filled jugular veins.

side of the N-*myc*$^{9a/BRP}$ embryo (Fig. 5B). The lungs begin their development by budding from the trachea into the surrounding mesenchyme and are visible as two pouches at 10.5 days of development. This early phase of lung development occurred normally in N-*myc*$^{9a/BRP}$ embryos (not shown).

In order to determine whether aspects of lung development other than the branching of the lung epithelium were affected by the reduction in N-Myc protein levels, we performed RNA in situ hybridizations on wild-type and N-*myc*$^{9a/BRP}$ lungs at 12.5 days p.c. (Fig. 6). *Flk-1*, which encodes a tyrosine kinase receptor for vascular endothelial
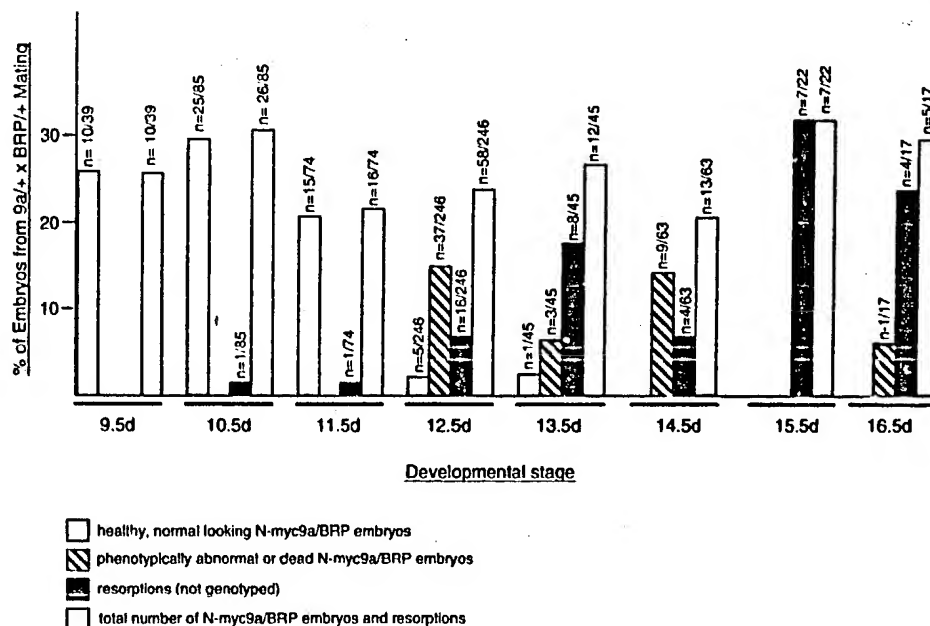
Fig. 3. Percentage of embryos in a N-*myc*$^{9a/+}$×N-*myc*$^{BRP/+}$ litter that are N-*myc*$^{9a/BRP}$, as a function of developmental stage. N-*myc*$^{9a/BRP}$ embryos are separated into two groups: (1) healthy, phenotypically normal embryos (stippled bars), and (2) phenotypically abnormal or dead embryos (hatched bars). Resorption sites visible in the uterus but not typed with respect to N-*myc* are included as a separate category (black bars). Note that the total of resorptions, abnormal N-*myc*$^{9a/BRP}$ embryos and normal N-*myc*$^{9a/BRP}$ embryos adds up to approximately 25% of the offspring at each time point (white bars). Numbers are given as a fraction of the total number of embryos dissected from N-*myc*$^{9a/+}$×N-*myc*$^{BRP/+}$ crosses at that developmental stage.
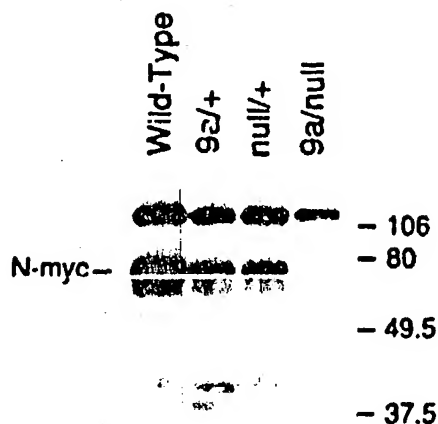
Fig. 4. Western analysis of N-Myc protein levels in extracts of 11.5 days p.c. embryos of the four possible types of offspring from an N-myc$^{9a/+}$×N-myc$^{BRP/+}$ cross. Each lane contains approximately 20 µg of protein. The N-Myc protein runs at approximately 65×10³ M_r. A cross-reacting band appears at approximately 100×10³ M_r.

growth factor, marks endothelial cells in the developing capillaries and blood vessels in the mouse (Yamaguchi et al., 1993; Millauer et al., 1993), and as such is expressed in a punctate manner throughout the lung mesenchyme where the lung vasculature is developing (Fig. 6C). The pattern and intensity of flk-1 expression was not affected in N-myc$^{9a/BRP}$ lungs (Fig. 6G), indicating that the development of the lung vasculature is not affected by the reduction in N-Myc protein observed in N-myc$^{9a/BRP}$ embryos. Fig. 6B,F shows the pattern of expression of N-myc in wild-type and N-myc$^{9a/BRP}$ lungs, respectively, at 12.5 days p.c., reconfirming that expression is restricted to the lung epithelium and showing that the reduced N-Myc protein levels in N-myc$^{9a/BRP}$ embryos did not lead to a complete loss of N-myc-expressing cells in the lung epithelium. N-myc$^{9a/BRP}$ lungs appeared to express more than 15% of normal levels of N-

myc RNA (Fig. 6F). This is because the N-myc^{BRP} allele encodes a transcript that includes the entire N-myc open reading frame, but which contains stop codons that prevent translation of any part of the N-myc protein (Stanton et al., 1990). Fig. 6D,H demonstrate that levels of c-myc mRNA at the cellular level were not affected by the reduction in N-Myc protein in the lungs of N-myc$^{9a/BRP}$ embryos. C-myc is normally expressed in the lung mesenchyme (Fig. 6D, Hirning et al., 1991) and the pattern and level of c-myc expression was not altered in the N-myc$^{9a/BRP}$ lung, despite its reduced size (Fig. 6H).

Heart development

The other clearly visible defect in N-myc$^{9a/BRP}$ embryos examined at 12.5 days p.c. was in the morphology of the heart. While N-myc$^{9a/BRP}$ hearts had the normal four-chamber structure, normal endocardial cushions, valves and septa, the heart was small and the myocardium was abnormally thin (Fig. 7A-D). The latter defect was particularly apparent in the 'compact', or subepicardial layer of the ventricular myocardium while the atrial myocardium and the inner trabecular layer of the ventricles were less severely affected. In mammals, trabeculation occurs early during heart development and, at later stages, growth occurs largely in the compact layer (Rumyantsev, 1991). In N-myc$^{9a/BRP}$ embryos, this growth of the compact layer appeared not to occur, so that the N-myc$^{9a/BRP}$ myocardium at 12.5 days p.c. was no thicker than it had been at 10.5 days p.c.

Occasionally, N-myc$^{9a/BRP}$ embryos survived until 14.5 days p.c. These embryos were always grossly abnormal, with large edemas in the neck area, and were smaller than their littermates. Fig. 7E and F compares wild-type and N-myc$^{9a/BRP}$ hearts at this stage, showing that the compact layer of the myocardium of the compound heterozygote was still no more developed than it had been at 10.5 days.

It seems likely that this failure in the proliferation of the ventricular myocardium leads to a failure of the circulatory system and ultimately in fetal death. The inefficient function of the heart was demonstrated by the hugely expanded

Fig. 5. Comparison of lung development in wild-type (A) and compound heterozygote N-myc$^{9a/BRP}$ (B) embryos at 12.5 days p.c. Comparable frontal sections were taken at the level of the right postcaval lobe. E, esophagus; L, left lung; RP; right lung, postcaval lobe. Bar, 200 µm.
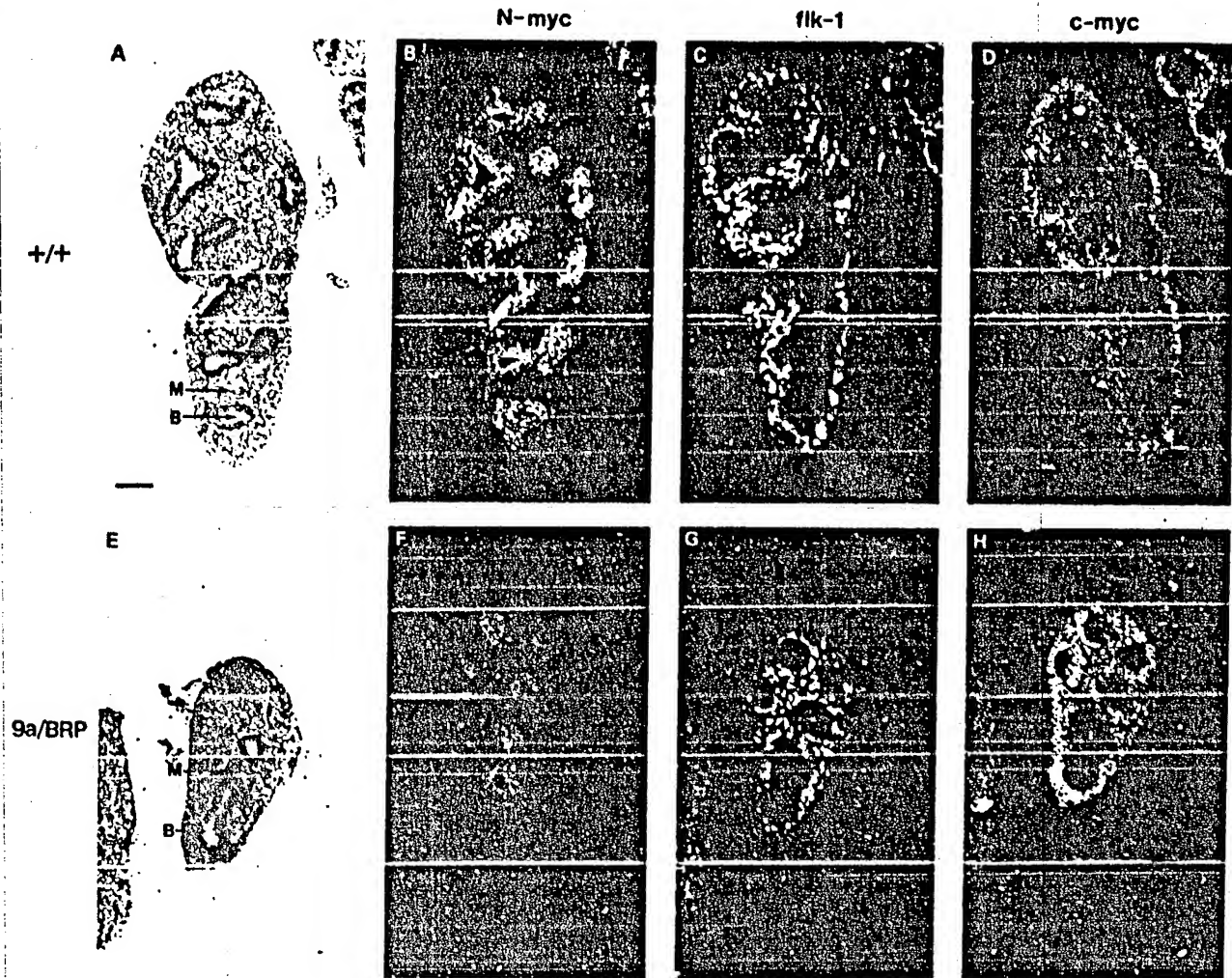
Fig. 6. Expression of N-*myc*, *flk-1*, and c-*myc* in wild-type and N-*myc*[9a/BRP] lungs at 12.5 days p.c.. (A,E) Bright-field photomicrographs of sagittal sections of the left lung of +/+ (A) and compound heterozygote N-*myc*[9a/BRP] (E) embryos. Wild-type (B-D) and N-*myc*[9a/BRP] (F-H) serial sections hybridized to the RNA probes shown, were photographed in dark field. B, bronchiole; M, lung mesenchyme. Bar, 100 μm.

jugular veins of N-*myc*[9a/BRP] embryos (Fig. 7G,H), since the lack of a highly muscularized ventricular myocardium pumping blood from the heart could lead to a back-up of blood in the major veins. However, it should be noted that we did not see this marked distension in the other major veins that lead into the heart.

We wished to determine whether the observed defect in the development of the compact layer of the ventricular myocardium correlated with N-*myc* expression in the heart. Expression of N-*myc* in the heart at 9.5 days p.c. has been described (Katoh et al., 1991), and we have confirmed this by whole-mount RNA in situ hybridization (data not shown). However the differentiation of trabecular and compact layers has only just begun at this stage, and N-*myc* expression was not shown to be restricted to one specific layer. Our experiments indicate that, by 10.5 days p.c., N-*myc* expression in the heart is largely confined to the compact layer and not to the trabecular layer of the devel-

oping ventricular myocardium (Fig. 8B). For comparison, α-cardiac actin, a marker of differentiated myocytes in the developing heart (Sassoon et al., 1988), is expressed in both the trabeculae and the compact layer (Fig. 8C). We observed continued N-*myc* expression in the compact layer at the time when a mutant phenotype was first observed in the heart (12.5 days p.c., Fig. 9B,G). This higher level of of N-*myc* expression in the compact layer compared to the trabecular layer is consistent with a direct role in the development of the mutant phenotype we have observed in N-*myc*[9a/BRP] embryos.

Considerable N-*myc* expression was detected in the compact layer of 12.5 days p.c. N-*myc*[9a/BRP] hearts (Fig. 9B,G) because the N-*myc*[BRP] mutation prevents translation but not transcription of N-*myc*, as noted above. However, as expected, the layer of N-*myc*-expressing cells was narrower in the ventricular myocardium of compound heterozygotes than in the wild-type myocardium (compare Figs 9B and
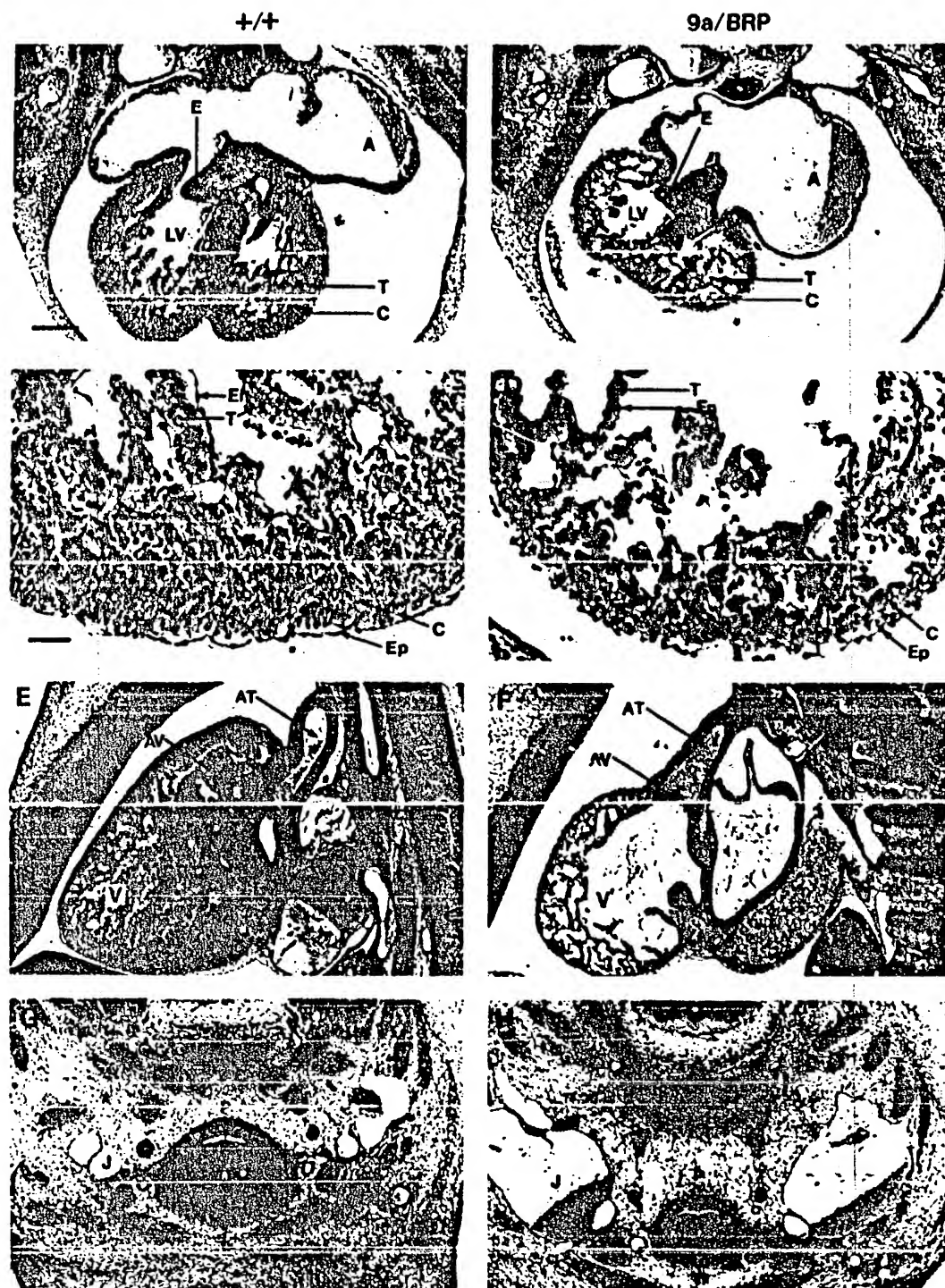
Fig. 7. Comparison of heart development in wild-type and compound heterozygote N-*myc*[9a/BRP] embryos at 12.5 and 14.5 days p.c.
Left-hand photographs show wild-type embryos, and right-hand photographs show N-*myc*[9a/BRP] embryos. (A,B) Low-power
magnification photomicrographs of frontal sections of 12.5 days p.c. hearts taken at the level of the left atrioventricular canal.
(C,D) High-power magnification of the ventricle of 12.5 day hearts. (E,F) Sagittal sections of 14.5-day hearts. (G,H) Frontal sections
through the jugular veins at 12.5 days. A, atrium; AT, aortic trunk; AV, aortic valve; C, compact layer; E, endocardial cushion; En,
endocardium; Ep, epicardium; J, jugular veins; LV, left ventricle; P, pharynx; V, ventricle; T, trabeculae; Tr, trachea. For A,B,E,F,G,H:
bar, 250 μm; for C,D: bar, 50 μm.
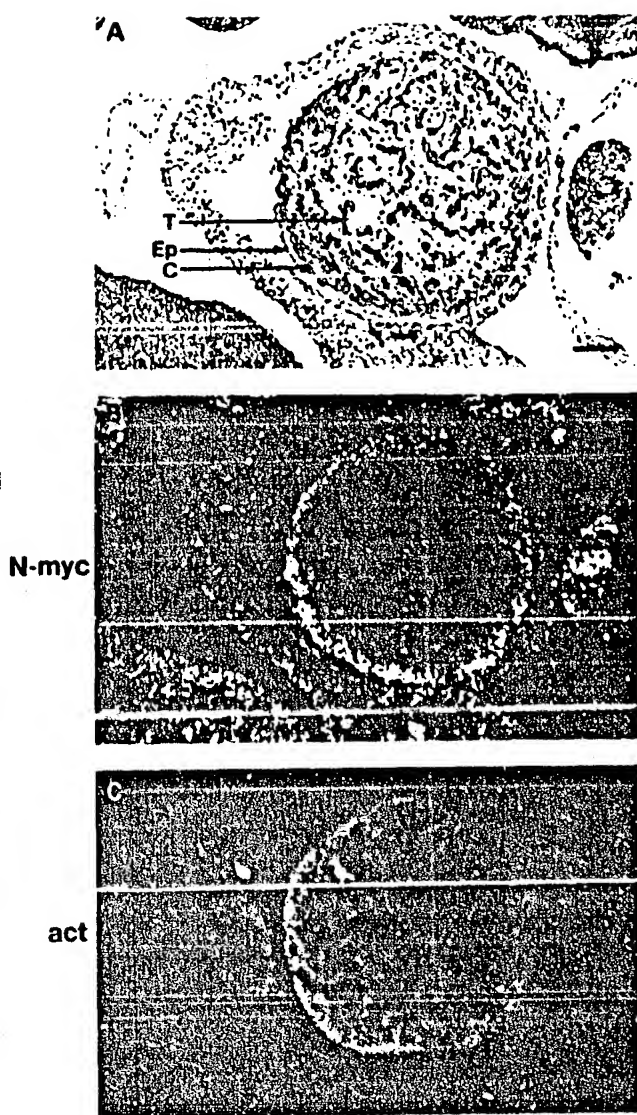
Fig. 8. Expression patterns of N-*myc* and α-cardiac actin in the heart at 10.5 days p.c. (A) Bright-field photomicrograph of a hematoxylin and eosin-stained parasagittal section of a wild-type ventricle. (B,C) Dark-field photomicrographs of serial sections showing N-*myc* (B) and α-cardiac actin (C) expression by RNA in situ hybridization. C, compact subepicardial layer; T, trabeculae; Ep, epicardium. Bar, 100 μm.

8B). The observation that the mutant alleles continue to be transcribed in the N-*myc*$^{9a/BRP}$ myocardium suggests that cells that normally express N-*myc* are present but in reduced numbers.

Flk-1, the endothelial cell marker described above, is expressed in the endocardium (Yamaguchi et al., 1993), which is the mesodermally derived inner lining of the heart that later contributes to the endocardial cushion and heart valves through an epithelial-to-mesenchymal transition (Fig. 9D,I; Markwald et al., 1990). The differentiation of the endocardium was not affected in N-*myc*$^{9a/BRP}$ embryos, as

demonstrated by the continued expression of *flk-1* in the endocardium (compare Figs 10D,I and 8D,I). The N-*myc*$^{9a/BRP}$ genotype also did not prevent the differentiation of cardiac myocytes in the myocardium, as indicated by the strong expression of α-cardiac actin in both the compact and trabecular layers of the mutant myocardium (compare Figs 10C,H and 9C,H).

The finding that reduction in N-*myc* expression in the compact layer of the heart caused a myocyte hypoplasia evident at 12.5 days p.c. was interesting, in that overexpression of the closely related c-*myc* proto-oncogene in the heart of transgenic mice has been described to cause a hyperplasia of the myocytes which is also evident during development (Jackson et al., 1990). The normal expression pattern of c-*myc* in the heart has not been described, so the relative roles of these two genes in myocyte proliferation in vivo was not clear. Fig. 9E,J demonstrates that c-*myc* is expressed at low levels in the heart, and that its pattern of expression is different, and to some extent complementary to that of N-*myc*. While N-*myc* is expressed in the compact layer of the ventricular myocardium, c-*myc* expression is largely restricted to cells adjacent to and on the outside of the compact layer, and to isolated cells or clusters of cells adjacent to and on the inside of the compact layer. The expression on the outer surface of the heart is similar to that of *flk-1* (Fig. 8D,I) and probably represents expression in the capillaries that run between the myocardium and epicardium (Viragh and Challice, 1981). The small foci of grains on the inner surface of the myocardium also appears to represent c-*myc* expression in endocardial cells, as they are found specifically in the vicinity of red-blood-cell-containing capillaries. c-*myc* mRNA was also observed in endothelial cells lining the endocardial cushion. c-*myc* is only expressed at low levels in the myocardium itself, in spite of the fact that when it is expressed ectopically in the myocardium it causes myocytic hyperplasia (Jackson et al., 1990). In N-*myc*$^{9a/BRP}$ hearts, the expression of c-*myc* did not appear to be affected (Fig. 10E,J).

Other tissues

Careful examination of N-*myc*$^{9a/BRP}$ embryos between 10.5 and 14.5 days p.c. gave no evidence of defects in the kidney or brain, both of which are major sites of N-*myc* expression in the embryo, and both of which are affected in N-*myc*$^{BRP/BRP}$ embryos (Stanton et al., 1992; Charron et al., 1992). In four N-*myc*$^{9a/BRP}$ embryos that survived until 14.5 days p.c., the kidneys were small but were structurally normal (not shown). N-*myc*$^{BRP/BRP}$ mice have also been described as having reduced genital ridges (Stanton et al., 1992) and cranial and spinal ganglia. However, these structures were indistinguishable in N-*myc*$^{9a/BRP}$ embryos from those of their wild-type littermates (not shown).

DISCUSSION

We have generated mice that carry two different mutant alleles of the N-*myc* proto-oncogene in order to identify functions for N-*myc* that were not revealed in mice homozygous for each mutation individually. The N-*myc*^{9a} allele is a leaky mutation which in the homozygous condition causes perinatal lethality due to a defect early in lung branching
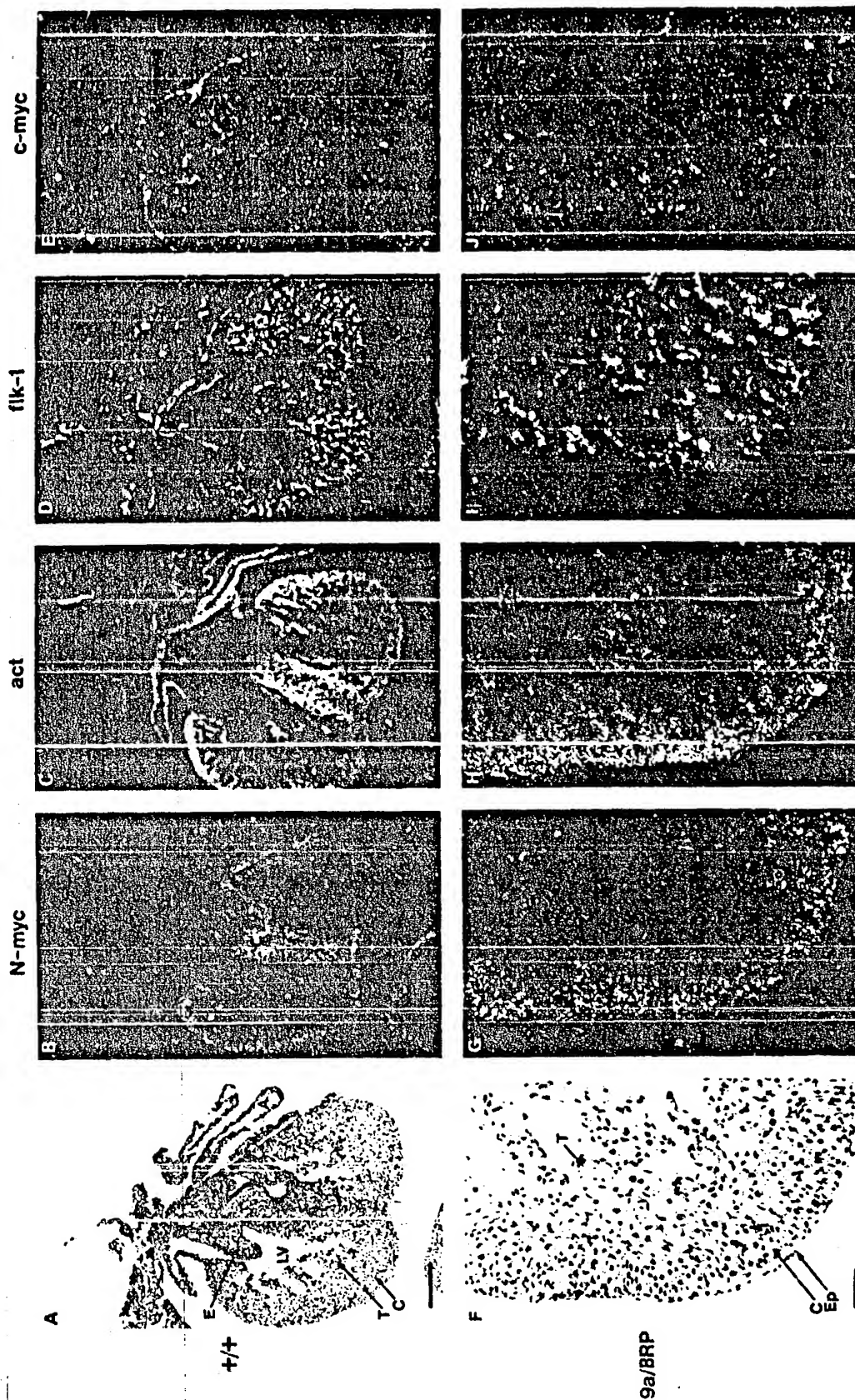
Fig. 9. Expression of N-*myc*, α-cardiac actin, *flk-1* and c-*myc* in the wild-type heart at 12.5 days p.c. (A) Bright-field photomicrograph of a hematoxylin and eosin-stained frontal section in the region of the left atrioventricular canal. (F) High power magnification of the section shown in a, through the ventricle. (B-E) Dark-field photomicrographs of serial sections hybridized to the RNA probes shown above. (G-J) High power detail of the sections shown in B-E. Act, α-cardiac actin; A, atrium; C, compact layer; E, endocardial cushion; Ep, epicardium; LV, left ventricle; T, trabeculae. For A-E, bar, 200 μm; for F-J, bar, 50 μm.
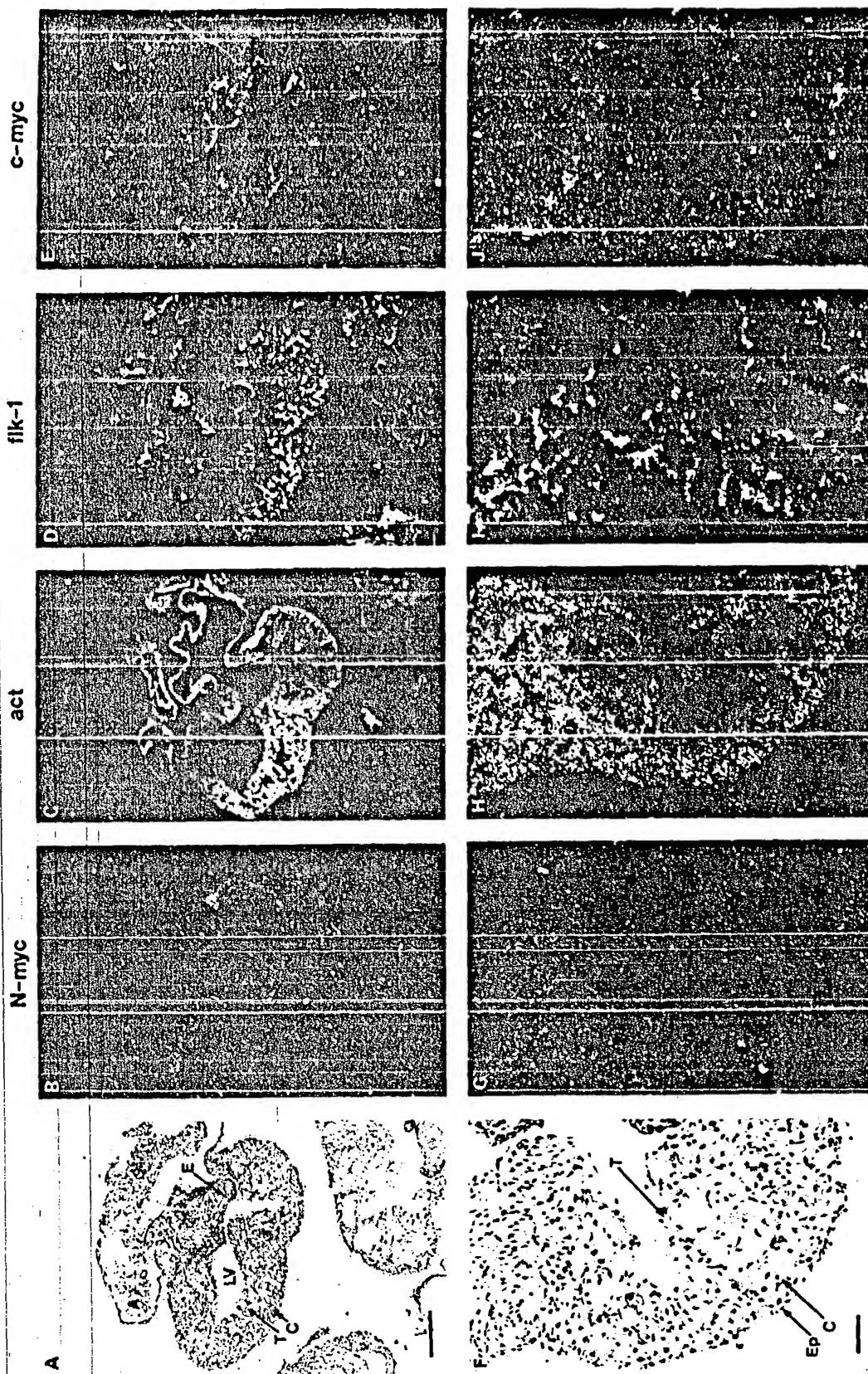
Fig. 10. Expression of N-*myc*, α-cardiac actin, *flk-1* and c-*myc* in compound heterozygote N-*myc*⁹ᵘ/BRP heart at 12.5 days p.c. (A) Bright-field photomicrograph of a hematoxylin and eosin-stained frontal section in a region comparable to that shown in Fig. 9. (F) High power magnification of the section shown in A, through the ventricle. (B-E) Dark-field photomicrographs of serial sections hybridized to the RNA probes shown. (G-J) High power detail of the sections shown in B-E. Act, α-cardiac actin: A, atrium; C, compact layer; E, endocardial cushion; Ep, epicardium; LV, left ventricle; T, trabeculae. For A-E, bar, 200 μm, for F-J, bar, 50 μm

morphogenesis. The levels of residual N-Myc protein (approximately 25% of normal levels) are presumably sufficient to support the normal development of other tissues in which N-*myc* normally functions. The N-*myc^BRP* allele is likely to be a mutation which, in the homozygous condition, results in embryonic lethality at approximately 11.5 days p.c., at which point the epithelial component of the mesonephros, brain, lung, stomach and intestine are all hypoplastic (Stanton et al., 1992). The phenotype of N-*myc^9a/BRP* compound heterozygotes is intermediate to the two homozygous mutant phenotypes, consistent with the observation that the N-*myc^9a/BRP* heterozygotes contain lower levels of N-Myc protein than are present in N-*myc^9a/9a* embryos. N-*myc^9a/BRP* embryos die between 12 and 15 days p.c., and a number of tissues that are affected in N-*myc^BRP/BRP* homozygotes, such as the kidney, brain and cranial and spinal sensory ganglia, appear to be normal. The lungs, which are the main organ affected in N-*myc^9a/9a* homozygotes, are even more severely affected in N-*myc^9a/BRP* embryos. Compound heterozygotes also showed a defect in the development of the compact subepicardial layer of the heart, and appeared to die from a failure of heart function caused by a hypoplasia of ventricular myocytes in the compact layer. These results indicate a critical role for N-*myc* in the development of the heart.

N-*myc* in heart development

The reduced levels of N-Myc protein found in N-*myc^9a/BRP* embryos result in a considerable thinning of the subepicardial compact layer of the myocardium by 12.5 days of development. Consistent with this phenotype, N-*myc* expression is expressed much more strongly in the compact layer of the heart at 10.5 and 12.5 days p.c. than in the trabecular layer of the myocardium or in the endothelium. Trabeculation, or formation of the myocardial projections that form a lattice of contractile cells throughout much of the ventricles of the embryonic heart, occurs early during heart development in the mouse, beginning around day 9.5 of gestation (Challice and Viragh, 1973). By a number of ultrastructural and cytochemical criteria, the myocytes within the trabeculae are more highly differentiated than the myocytes in the compact layer (Rumyantsev, 1991). Thus compact layer myocytes are more basophilic and richer in RNA, while trabecular myocytes contain more mitochondria, ribosomes and granular endoplasmic reticulum. Myofibrils in trabecular myocytes are thicker and more highly organized than in compact layer myocytes, where myofibrils are present but are scattered randomly relative to one another in the cytoplasm. Consistent with this picture is the observation that the rate of cell proliferation in the compact layer is 2- to 3-fold higher than in the trabeculae (Rumyantsev, 1977; Tokuyasu, 1990). The highly differentiated myocytes in the trabecular layer have been postulated to be responsible for the early beating of the heart while, at later stages, the thickened compact layer becomes the major contractile force. The cardiac hypoplasia that we observe in N-*myc^9a/BRP* embryos is more apparent in the compact layer. This may explain our observation that embryonic lethality does not occur until later during heart development, when heart function may depend more on the compact layer than on the trabecular layer.

Our observation of N-*myc* expression in the compact layer and the absence of development in the compact layer in N-*myc^9a/BRP* mice suggests that N-*myc* is required either for the proliferation of myocytes in the compact layer, and/or for preventing the differentiation of these cells, although there are other possible explanations, such as that they are dying prematurely. The simplest explanation is the former. However, the second hypothesis, in which N-*myc* expression prevents the terminal differentiation of compact layer myocytes into trabecular-type myocytes, is consistent with previous descriptions of N-*myc* expression in the embryo, in which N-*myc* expression is correlated with cells in an undifferentiated state in the kidney, brain and skin, regardless of their proliferative state, and the further differentiation of these cells is correlated with down-regulation of N-*myc* (Mugrauer et al., 1988).

In their description of embryos homozygous for a null mutation in N-*myc*, Charron et al. (1992) noted a defect in the development in the heart, visible as early as 9.5 days p.c., in which development was apparently slowed as evidenced by the absence of endocardial cushion tissue and of interatrial and interventricular septa. It was postulated that N-*myc* is involved in the generation of the inductive signal sent by the myocardium to the endocardium to induce the epithelial-to-mesenchymal transition of the endocardium, which forms these anlagen of the cardiac valves and septa. Our results confirm a function for N-*myc* in the development in the heart, but tend to support a role for N-*myc* within the myocardium itself, although this could presumably have a secondary impact on endocardial differentiation in more severe mutants. We have not observed defects in the formation of septal or endocardial cushion tissue in N-*myc^9a/BRP* embryos.

c-*myc* in heart development

When c-*myc* is overexpressed in the heart of RSV/c-*myc* transgenic mice, these mice develop a fetal cardiac myocyte hyperplasia and at birth have more than twice the normal number of cardiac myocytes (Jackson et al., 1990). This has suggested that endogenous c-*myc* may play a role in cardiac myocyte proliferation in vivo. However, we have demonstrated that c-*myc* is only expressed at low levels in the heart at 12.5 days p.c. and that this expression is largely in endothelial cells and not in the myocardium. Background levels of c-*myc* expression in the heart were also observed in mouse embryos at 13.5 days p.c. (Stanton et al., 1992) and in first trimester human embryos (Pfeifer-Ohlsson et al., 1985). N-*myc*, in contrast, is expressed at high levels in the compact layer of the ventricular myocardium at 10.5 and 12.5 days. Combined with the hypoplasia of the myocardium that we have observed in N-*myc^9a/BRP* embryos, these data suggest that N-*myc* rather than c-*myc* may play a primary role in the regulation of proliferation and/or differentiation of ventricular myocytes during heart development, and that the effect of c-*myc* in these transgenic mice may reflect the possibility that in this instance c-*myc* can mimic the normal effects of N-*myc*. It has previously been observed that different *myc* family genes can cause the same tumour types in transgenic mice when they are overexpressed using identical promoters, even though this expression may be ectopic (Rosenbaum et al., 1989; Dildrop

et al., 1989; Adams et al., 1985). Furthermore, high levels of expression of one *myc* gene in transgenic mice can repress transcription of itself and of other *myc* genes (Rosenbaum et al., 1989; Dildrop et al., 1989; Adams et al., 1985). These results have suggested that at high levels, the various *myc* genes may be able to mimic each other's effects on downstream targets. This hypothesis has been strengthened by the observations that N-Myc and c-Myc proteins bind the same core DNA sequence in vitro, and that N-, L- and c-*myc* all form heterodimers with Max in vivo (Blackwood et al., 1992; Wenzel et al., 1991; Mukherjee et al., 1992), and all transform cells in culture through their interaction with Max (Mukherjee et al., 1992). We are presently crossing RSV/c-*myc* mice with the N-*myc* mutant mice to generate N-*myc*$^{9a/BRP}$ embryos that also express this c-*myc* transgene in the heart. If c-*myc* in these transgenics truly mimicks the normal role of N-*myc*, the cardiac myocyte hypoplasia of compound heterozygous embryos is expected to be rescued by the transgene.

Recently, Davis et al. (1993) have described the phenotype of mice that bear a null mutation in c-*myc*. These embryos die before 10.5 days p.c. and exhibit, among other abnormalities, an enlargement of the heart and a dilated, fluid-filled pericardium. This is unexpected in light of the observation, described above, that mice with ectopic expression of c-*myc* in the heart have enlarged hearts. However, it is still unclear whether the heart abnormality in the c-*myc* null mutants is a direct result of the mutation or is secondary to other defects that are causing the embryo to die.

In spite of the possibility that c-*myc* expression may be able to replace N-*myc* function, we observe no up-regulation of c-*myc* in either the compact layer of the myocardium or in the lung epithelium of N-*myc*$^{9a/BRP}$ mice. This may be because cross-regulation of the *myc* family genes does not normally occur in these tissues as it does when they are over-expressed in transformed cells or in transgenic mice. Stanton et al. (1992) showed that c-*myc* is expressed in the telencephalon of N-*myc*$^{BRP/BRP}$ embryos, and this observation was interpreted to indicate cross regulation of N-*myc* and c-*myc* in the neuroepithelium.

N-*myc* in lung development

Mice homozygous for the N-*myc*^{9a} mutation die at birth due to a defect in lung morphogenesis which is visible as early as 12.5 days p.c. (Moens et al., 1992). We have postulated that N-*myc* is required for the lung epithelium to respond to local inductive signals emanating from the lung mesenchyme, which cause branching to occur. N-*myc*$^{9a/BRP}$ embryos have more severely affected lungs, with only a rudimentary branching pattern at 12.5 days. However, the earliest events of lung development, in which two buds are induced to grow from the trachea by surrounding mesenchyme (Spooner and Wessells, 1970), occur normally in N-*myc*$^{9a/BRP}$ and indeed in N-*myc*$^{BRP/BRP}$ embryos (Stanton et al., 1992). We and others (Hirning et al., 1991; Moens et al., 1992) have shown that N-*myc* is expressed in the lung epithelium and, further, that expression is largely restricted to bronchioles and is present at very low levels in the trachea and bronchi. These results suggest that N-*myc* is involved in branching morphogenesis in the lung but not in the initial

induction of budding of the tracheal epithelium. Experimental manipulations in vitro have suggested that there are different mechanisms for budding versus branching of the lung epithelium. A number of different stimuli, including salivary gland mesenchyme and bronchial mesenchyme, can induce supernumerary buds in tracheal epithelium, but only bronchial mesenchyme is able to induce those buds to branch (Wessells, 1970; Spooner and Wessells, 1970). The phenotypes of mice bearing mutations in N-*myc* provide genetic evidence for such a mechanism for lung development and provide a candidate gene that is involved in the control of one process (branching) and not the other (budding).

Tissue-specific effects of N-myc mutant alleles

Only a subset of the tissues that normally express N-*myc* are visibly affected in N-*myc*$^{9a/BRP}$ embryos. N-*myc*$^{9a/9a}$ embryos have approximately 25% of wild-type levels of N-Myc protein and, in these embryos, the lungs and spleen are the only tissues affected (Moens et al., 1992). N-*myc*$^{9a/BRP}$ embryos have approximately 15% of wild-type levels of N-Myc protein and, in these embryos, the heart is also affected. However, 15% of normal levels of N-Myc protein appear to be sufficient for normal genitourinary and nervous system development. The molecular basis for this remains to be determined. It is possible that different tissues within the embryo make different amounts of normal protein relative to the amounts in wild-type embryos. We have previously attempted to correlate the relative levels of normal N-*myc* mRNA in different tissues of N-*myc*$^{9a/9a}$ embryos with the presence or absence of a mutant phenotype (Moens et al., 1992) and, although there were differences among the tissues examined, no strong correlation could be established. Another explanation for the tissue-specific effects of N-*myc* mutant alleles is that different tissues are affected differently by approximately the same reduction in N-Myc protein because of differences in the ratio of N-Myc protein to Max (Blackwood and Eisenman, 1991), to Max-associated proteins such as Mad and Mxi1 (Ayer et al., 1993; Zervos et al., 1993), or to other Myc proteins.

Myc proteins require dimerization with Max for DNA binding (Blackwood and Eisenman, 1991; Prendergast and Ziff, 1991; Kato et al., 1992). A number of lines of evidence have suggested that Max overexpression can inhibit transformation (Mukherjee et al., 1992; Makela et al., 1992; Prendergast et al., 1992) and transactivation (Kretzner et al., 1992; Amati et al., 1992) by *myc* genes. Mad, cloned by virtue of its ability to dimerize with Max, has been shown to compete with Myc for binding to Max, and to thereby inhibit transactivation by Myc (Ayer et al., 1993). Mxi1 (Zervos et al., 1993) and other, as yet unidentified, Max partners presumably act in a similar manner and are also likely thereby to inhibit N-*myc* function. Also, L-Myc, a poorly transforming member of the Myc family, has been shown to prevent transformation by other *myc* genes, presumably by competing for and forming less active DNA-bound complexes with Max (Mukherjee et al., 1992). In cell types where a number of Max-associated and Myc proteins compete with N-Myc protein for dimerization with Max and sites on DNA, an 85% reduction in N-Myc protein is expected to reduce the response of downstream targets of N-

Myc more strongly than in cells where there are no competitors for Max binding. Interestingly, L-*myc* is co-expressed with N-*myc* in the lung, perhaps in the same cell type (Zimmerman et al., 1986), but the two genes are not co-expressed in the ...dney (Mugrauer and Ekblom, 1991), where neither the N-*myc*[9a/9a] nor the N-*myc*[9a/BRP] embryos have an abnormal phenotype. It will be interesting to compare the detailed expression patterns of the various interacting factors with the tissues affected by the N-*myc* mutations.

Conclusions

We have generated a third N-*myc* mutant phenotype by combining leaky and null alleles in a compound heterozygote. These mice have allowed us to study the function of N-*myc* at a stage in development that is not reached in embryos homozygous for the null allele (Stanton et al., 1992) and that is not affected in embryos homozygous for the leaky allele (Moens et al., 1992). Classical genetic studies of development in a number of systems have demonstrated the importance of studying the phenotypic effects of different mutant alleles and combinations of mutant alleles in a given gene in order to determine its multiple roles in the course of development. Our results have shown that the technique of gene targeting by homologous recombination in the mouse can be used to the same ends. The clear delineation of a function for N-*myc* in both lung branching morphogenesis and myocardial development also provides target tissues in which to search for the elusive downstream genes in the N-*myc* signaling pathway.

Note added after acceptance

Recently, a third description of embryos homozygous for a putative null allele of N-*myc* has been published (Sawai et al., 1993). The overall phenotype of these mutant embryos is very similar to those described by Stanton et al. (1992) and Charron et al. (1992), but the defect in heart development is shown to be primarily in the myocardium.

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REVIEW

AV

Randall Wade Moreadith · Nina Butwell Radford

Gene targeting in embryonic stem cells: the new physiology and metabolism

Abstract The development of transgenic technology, whereby genes (or mutations) can be stably introduced into the germline of experimental mammals, now allows investigators to create mice of virtually any genotype and to assess the consequences of these mutations in the context of a developing and intact mammal. In contrast to traditional "gain-of-function" mutations, typically created by microinjection of the gene of interest into the one-celled zygote, gene targeting via homologous recombination in pluripotential embryonic stem cells allows one to modify *precisely* the gene of interest. The purpose of this review is to introduce the reader to the history of development of embryonic stem cell technology, the current methods employed to create "knock-out" mice, and the application of these methods to solve problems in biology. While the technology promises to provide enormous insight into mammalian development genetics, our desire is that this review will stimulate the application of gene targeting in embryonic stem cells to begin to unravel problems in complex regulatory pathways, specifically intermediary metabolism and physiology.

Key words Gene targeting · Transgenic animals · Molecular biology · Metabolism · Physiology

Abbreviations *EC cell* Embryonal carcinoma cell · *ES cell* Embryonic stem cell · *HPRT* Hypoxanthine phosphoribosyltransferase · *NMR* Nuclear magnetic resonance

Introduction

The development of techniques for introducing genes (or mutations) stably into the germline of experimental

R.W. Moreadith (✉) · N.B. Radford
Molecular Cardiology Laboratories, 5323 Harry Hines Boulevard.
University of Texas Southwestern Medical Center, Dallas,
TX 75235-8573, USA

Present address:
¹ Quintiles, Inc., 1007 Slater Road, Morrisville, NC 27707, USA

mammals [1], referred to as "transgenic technology," has provided unique insight into complex biologic phenomena. Although simplistic, this technology can now be broadly defined into two experimental categories: "gain-of-function" mutations, typically created by microinjection of the gene (transgene) of interest directly into the zygote stage of development (for example, to define the controlling elements for a muscle-specific gene), and "loss-of-function" mutations, which employ embryonic stem (ES) cells. The technique of DNA microinjection results in *random integration* of the transgene, giving rise to the founder animal(s). The founder animals are then bred individually to establish independent lines of transgenic animals that can undergo further characterization [e.g., pattern(s), levels and consequences of expression of the transgene]. However, many of these lines exhibit variable expression of the gene of interest since the transgene may integrate in a manner that alters its expression (for example, by integration near controlling elements that affect the pattern and level of expression), and this may confound the interpretation of results.

In contrast, the development of gene targeting via homologous recombination in pluripotential ES cells allows one to modify *precisely* the gene of interest. It is now possible to create mice of virtually any genotype, and to assess the consequences of these mutations in the context of a developing and intact mammal. This technology, which is typically used to create the null genotype ("knock-out" mice), has frequently provided the definitive experimental evidence regarding the functions of the encoded proteins. However, in many instances these mutations have changed the prevailing notions. For example, gene targeting at the endothelin loci subsequently led to the creation of mice with Hirschsprung's disease (aganglionic megacolon [2]) instead of the anticipated phenotype (abnormal control of blood pressure). Indeed, if one had even predicted these mice would survive the absence of a cellular gene that is so widely expressed, one might have been in the minority!

The purpose of this review is to introduce the reader to the history of development of ES technology, the cur-

rent methods employed to create "knock-out" mice, and the application of these methods to solve problems in biology. While the technology promises to provide enormous insight into mammalian development genetics, our desire is that this review will stimulate the application of gene targeting in ES cells to begin to unravel problems in complex regulatory pathways, specifically intermediary metabolism and physiology.

Historical development

Derivation and characterization of pluripotential stem cells

The development of gene-targeting technology in ES cells is an example of the convergence of classical cell biology with molecular biology. One of the seminal papers was published in 1975 by Mintz and colleagues at the Fox Chase Cancer Center [3]. It was well established that explantation of normal mouse embryos into extrauterine sites (typically the kidney capsule) subsequently led to the development of tumors known as teratocarcinomas. Cell lines could be derived from the tumors, passaged indefinitely in tissue culture, and when reintroduced into recipient mice gave rise to tumors with cell types representative of all germ layers (ectoderm, mesoderm, and endoderm). These properties suggested that these embryonal carcinoma (EC) cells were pluripotential, that is, capable of differentiating into a host of cell types under the appropriate conditions. In many instances these tumors contained cell types indistinguishable from the normal cellular counterpart in biochemical or ultrastructural detail. Thus, although these cells were considered to be "highly" malignant, it was quite clear that not all tissues derived from them displayed the malignant properties of the parental cells. Mintz and colleagues reasoned that these EC cells might in fact be totipotential – capable of contributing to the development of all *normal* tissues under the appropriate environmental cues "For this to occur, the initially malignant cells would presumably have to be brought into association with early embryo cells so that the latter could provide an organizational framework appropriate for normal development [3]." This was an astounding leap of logic at the time. She and her colleagues subsequently demonstrated that malignant EC cells, grown as an ascites tumor for over 8 years, could give rise to apparently normal mosaic mice upon introduction into normal blastocysts. Furthermore, the mosaic mice were capable of transmitting the EC cell genotype through the germline, that is, mating of these mosaic mice with wild-type partners gave rise to mice that were genotypically derived from the EC cells! These results, and those from many other laboratories, established the experimental basis for subsequent developments in the field. Indeed, Mintz precisely predicted the emergence of stem cell technology. "Thus, EC cells ... offer new possibilities for studying mammalian regulatory systems: the carcinoma cells

could first be experimentally mutagenized and selected during a brief in vitro sojourn and then cycled through mice via blastocyst injections. Participation in differentiation of a mosaic individual would permit developmental and biochemical analyses of the mutations; conversion of some cells to gametes would enable genetic analysis and mapping of the mutated regions through recombination and segregation during meiosis [3]."

These observations, made prior to the large-scale development of classical molecular biology (note that the above experiments were published prior to the development of DNA sequencing!) were quickly verified by many laboratories. However, working with EC cells proved to be quite cumbersome due to their propensity for aneuploidy, and the attention turned to isolation of pluripotential cells from *normal* embryos. Early attempts failed until 1981, when Evans and Kaufman [4] and Martin [5] independently described the growth and maintenance of euploid cells, derived from normal mouse embryos explanted in culture, that displayed pluripotential properties (hereafter referred to as ES cells). This was quickly followed by the demonstration in 1984 these cells were capable of giving rise to germline chimeras upon introduction into normal blastocysts [6]. The stage was set – one could grow normal, diploid ES cells in culture for multiple passages without loss of the ability to contribute to normal development. Furthermore, the cells contributed to the development of gametes at a high frequency (germline competence), and the haploid genomes of these cells were transmitted to the next generation. Thus, introduction of mutations in these cells offered the possibility of producing mice with a predetermined genotype.

Homologous recombination: introduction of precise mutations into resident genes

In an elegant series of papers using mammalian cell lines [7–10], based primarily on prior work in *Saccharomyces cerrivisiae*, it became clear that cloned DNA could be precisely altered in vitro, and when introduced into cells via a number of methods (infection, transfection) would homologously recombine with the resident gene and introduce the desired mutation at that site in the genome. In lower eukaryotes (yeast and *Neurospora*) recombination at the homologous locus is the favored reaction and can occur essentially without selection, but in higher eukaryotes (mouse and *Drosophila*) the frequency of homologous recombination is rare. This necessitates the use of *positive* selection; typically, these rare events are selected for by introduction of genes conveying resistance to otherwise toxic metabolites (hygromycin, neomycin). Note that the introduction of a positive selection marker gene is also frequently used to disrupt (mutate) the gene of interest. In addition, one can enhance the frequency of these events by employing the additional strategy of *negative* selection – most commonly performed by use of the thymidine kinase (*TK*) gene flanking the targeting vector. ES

210

A

1 kb

HSV-TK   PGK-neo

probe A

B
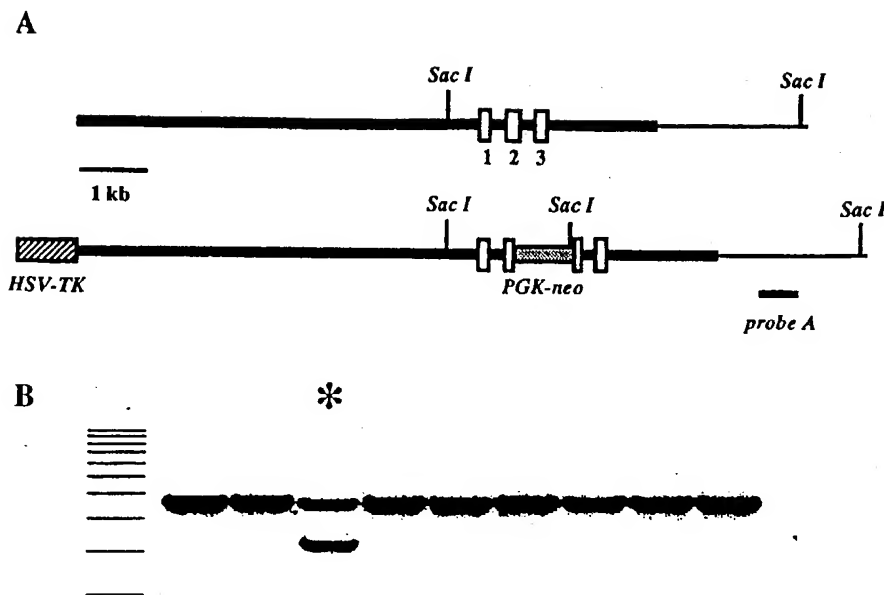
Fig. 1A, B Gene targeting at the murine cytochrome oxidase VIaH locus. A The structure of the gene, which has been reported elsewhere [12]. Briefly, the gene is comprised of three small exons (*open rectangles*). Exon 2 was disrupted with the expression cassette for neomycin phosphotransferase (*PGK-neo*), thus mutating the gene in its coding region and simultaneously providing positive selection. *Heavy lines*, the cloned vector used for transfection into J1 ES cells; note the targeting vector is flanked with the expression cassette for TK (*HSV-TK*) to allow for negative selection (see text). B Probe A was derived from a separate region of the gene (*thin line*) and was used to screen, by Southern analysis, DNA isolated from G418- and gancyclovir-resistant ES clones. Note the presence of the endogenous band at 5.7 kb in all clones, as well as the presence of the recombinant band at 4.2 kb (*asterisk*), in a *Sac*I digest of genomic DNA. Clone 3 was then used for blastocyst injection as illustrated in Fig. 2 to generate germline chimeras. *Bars* (*left*), 1-kb-size markers

cell clones which retain the *TK* gene (nonhomologous recombination) do not survive the addition of gancyclovir (or homologues) to the media due to the accumulation of toxic nucleosides, whereas cell clones which have undergone an authentic recombination lose the *TK* gene. This combined strategy is known as *positive-negative* selection [11] and routinely increases the targeting frequency by an order of magnitude or more.

An example of gene targeting in ES cells is illustrated in Fig. 1. The gene of interest in this example is a muscle-specific subunit of cytochrome oxidase (VIaH). The structure and regulation of the gene have recently been defined in the author's laboratory [12], but the precise function of this subunit, postulated to regulate the steady-state activity of cytochrome oxidase, remains largely unknown in the context of an intact animal. It is anticipated that creation of mice which lack this subunit will provide more insight into the precise role this subunit may play in the bioenergetics of cardiac and skeletal muscle.

Once these mutated ES cells are isolated as a pure clone, they can be introduced into the blastocoele cavity of a normal embryo, where they participate in the devel-

opment of all tissues and result in the production of chimeras (usually assessed at birth by acquisition of the dominant coat color phenotype Agouti). In subsequent matings of these chimeric mice, if the ES cells have contributed to formation of germ cells, the mutant gene is transmitted to their progeny. By mating heterozygotes, each harboring a mutated copy of the gene of interest (detected by analysis of the isolated DNA), one can derive embryos and mice which are homozygous for the mutation. These techniques are illustrated schematically in Fig. 2.

"Knockout mice" : the new genetics

The initial attempts at gene inactivation in murine ES cells took advantage of selection methods designed to reflect loss of an enzyme activity, hypoxanthine phosphoribosyltransferase (HPRT), following random integration of proviruses [13, 14]. Since the vast majority of ES cell lines used were male (male chimeric mice can be mated frequently, thus producing numerous offspring to assess germline competence), random integration of DNA (in this case retroviruses) would be expected to inactivate the single copy of the HPRT gene on the X chromosome at a low but detectable frequency and subsequently confer growth properties in a defined (hypoxanthine-aminopterin-thymidine) medium. These HPRT-minus cell lines were used to make mice deficient in HPRT, a potential mouse model of the human disorder Lesch-Nyhan syndrome [14, 15]. However, the absence of a neurologic phenotype in these mice was readily apparent, eventually precipitating a search for alternative explanations of why purine salvage might be different in the two species. This lead to the elucidation that adenine phosphoribosyltransferase was the major enzyme involved in purine salvage in the mouse. Indeed, when HPRT-deficient mice were exposed to inhibitors of adenine phosphoribosyltransfer-

Fig. 2 General strategy for creating "knock-out" mice

**129-Sv strain
(Agouti coat)
parental strain
for derivation of
ES cell**

**Pluripotential
embryonic stem
cell clones**

**Transfect with targeting vector
Select resistant clone**

**Isolate
blastocysts
from donor
(C57BL6)**

**Microinject into
blastocoel cavity
of donor embryos
Implantation
into recipient**

**Chimera, breed
with C57BL6**

**Presence of Agouti coat
color indicates germline transmission -
Analyze DNA for mutation**

ase, they developed neurologic phenotypes more representative of the human disorder [16]. The HPRT locus continued to be the subject of intense investigation, however, defining many of the parameters routinely employed now to target genes – for example, length and degree of homology to mediate highly efficient gene targeting [17, 18].

The first description of gene targeting via homologous recombination in murine ES cells was published in 1987 [19]. To date, several hundred novel mouse mutants – "knockout" mice – have been created, with dozens being reported monthly. A synopsis of these mutations is obviously beyond the scope of this review, but it is readily apparent from a perusal of these mutations that the

Table 1 Established methodologies for the study of murine physiology and metabolism

Methodology	Physiologic measure	References
Cardiovascular system		
Micromanometer catheter	LV hemodynamics	22–24
Indwelling arterial catheters	Blood pressure, heart rate	25–28
Reference microsphere and dilution	Cardiac output, regional blood flows, intravascular fluid volumes	26, 28
Echocardiography	LV mass, LV systolic function, wall motion abnormalities, heart rate	29–32
X-ray contrast microangiography	Ventricular volumes, ejection fraction	33
Swimming	Cardiac adaptations to chronic exercise	34
Langendorff perfusion	Intraventricular pressure, indices of LV contraction and relaxation, heart rate	35–37
Magnetic resonance imaging	Cardiac chamber sizes, coronary flow	38, 39
Magnetic resonance spectroscopy	pH, high energy phosphates	40
Tail-cuff sphygmomanometer	Blood pressure	27
Pulmonary system		
Plethysmography	Lung volumes, respiratory rate	41–44
Pulse oximetry	Arterial saturation	45
Pressure-volume curves	Elastic recoil	46
Forced oscillation	Pulmonary resistance	46
Methacholine challenge	Airway responsiveness	47
Skeletal muscle		
Skeletal muscle resection	Response to mechanical overload	48, 49
Magnetic resonance spectroscopy	pH, phosphocreatine, ATP, inorganic Phosphate, creatine kinase flux	50, 51
		49, 52, 53
Microvascular flow	Ischemia/reperfusion injury	54
Programmable treadmill	Fatiguability	55
Miscellaneous		
Magnetic resonance imaging	Knee joint degeneration	56
	Polycystic kidneys	57
	Renal edema, hepatic iron deposition	58
Magentic resonance spectroscopy	Hepatic ADP levels, creatine kinase flux	59
	Brain pH, free Mg, choline, N-acetylaspartate, creatine	60
Pathogen inoculation	Phagocyte oxidase function	61
Morris Water maze task, visible or hidden-platform tasks	Spatial learning	62, 63
Intruder test	Defensive aggression	64
Contextural and tone conditioning	Fear response	64
Swimming	Exercise-induced immunosuppression	65

technology has become one of the most powerful methods in the repertoire of approaches to gain insight into the functions of genes. To cite only a few applications, these include developmental biology, behavior and cognition, pharmaceutical research, and generation of models of human disease, such as cystic fibrosis and familial hypercholesterolemia [20, 21].

The new physiology and metabolism

Gene targeting in ES cells has only recently been applied to address problems in classical physiology and metabolism. Indeed, the creation of mutant animals, some of which have unpredictable and subtle phenotypes, has rekindled interest in developing techniques that allow one to characterize the animals precisely. This initiative, "molecular physiology," represents a new field of biology that addresses physiology and metabolism in the context of an intact animal harboring defined mutations in selected genes.

Established methodologies, such as light, immunofluorescence, and electron microscopy, are frequently used in postmortem tissue to describe histologic and ultrastructural changes in organs of interest in particular transgenic models of disease. Functional studies in the isolated, intact organ have also been accomplished, but it is becoming increasingly clear that studies of physiology in the intact animal may yield the greatest insight. A summary of methodologies which have been adapted to study murine cardiovascular, respiratory, and skeletal muscle physiology in vitro and in vivo is presented in Table 1. The remainder of our discussion focuses on examples of methodologies which have been developed to interrogate primarily cardiovascular phenotypes. Although most of these examples represent gain-of-function mutations, it is clear the methods can be applied to loss-of-function mutations as well.

Cardiovascular "molecular physiology"

At the present time ES cell technology exists only in the mouse (although see discussion below); thus creation of animals with anticipated cardiac phenotypes requires that

Optimally of course cardiovascular phenotypes would be interrogated in the intact animal. A number of invasive, catheter-based methods have been successfully applied to the mouse to make hemodynamic measurements in both the conscious and unconscious animal. Exciting advances have also been made in the application of non-invasive techniques such as magnetic resonance imaging and echocardiography to interrogate murine cardiac function.

The importance of the application of these methodologies to murine models of disease cannot be understated. Using a number of techniques outlined in Table 1, investigators have been able to characterize the cardiovascular phenotypes of a number of transgenic mice including lines which overexpress atrial natriuretic factor [25, 28], heat-shock proteins [36, 37, 40], human tissue kallikrein [27], β-adrenergic receptors [24], β-adrenergic receptor kinase and β-adrenergic receptor kinase inhibitor [23] as well as phospholamban deficient lines [32].

Summary

The advent of techniques to generate gain-of-function and loss-of-function mutations in laboratory animals represents one of the major accomplishments in cell and molecular biology in mammals over the past two decades. Although the technology is generally limited only to the mouse at present, substantial effort is underway to develop these techniques, and to refine existing techniques, in other species. Putative pluripotential ES cell lines have been derived in a number of other species including hamster [70], pig [71–75], sheep [73], cattle [76], rabbit [77], rat [78], mink [79], monkey [80], and even humans (81). Thus it seems likely the technology will be advanced into these additional species over the next few years, and each one of these may lend itself uniquely to problems ranging from development to tissue and organ physiology. Additionally, techniques such as those illustrated here and in Table 1 will need to be refined and applied to address each new mutation.

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Perspectives Series:
Molecular Medicine in Genetically Engineered Animals

Transgenesis in the Rat and Larger Mammals

Linda J. Mullins and John J. Mullins
Centre for Genome Research, The University of Edinburgh, Edinburgh EH9 3JQ, United Kingdom

Advances in biotechnology over the last ten years have made it possible for the researcher to alter gene expression in vivo in many diverse ways (1). With the establishment of embryonic stem (ES)[1] cell technology (2), more subtle and precise alterations can now be achieved than were previously possible using microinjection techniques. However, to date germline transmission has only been achieved with mouse ES cells, and microinjection continues to be the method most widely used for other species. While the mouse has a number of advantages, not least the depth of our knowledge of its genetics, other species are being increasingly used for transgenic studies due to their greater suitability for addressing specific questions. We will briefly review the application of transgenic technology to nonmurine species as it stands at present, with particular emphasis on developments appertaining to biomedical research.

Transgenesis by pronuclear injection

A number of significant limitations regarding the application of pronuclear injection to nonmurine animals have been identified (3), not least being the time and cost. Such limitations are due to longer gestation and generation times, reduced litter sizes, and higher maintenance costs. Further consideration must be given to the large numbers of fertilized eggs (and hence donor animals) required for microinjection, the high cost of carrying nontransgenic offspring to term, and the relatively low efficiency of gene integration. Such limitations are particularly severe for the production of bovine transgenics and, as a consequence, more significant departures from the standard procedures used for the mouse have been adopted for this species (4). For example, the use of in vitro embryo production in combination with gene transfer technology has played a large role in the development of transgenic cattle. The development of microinjected embryos through to the

Address correspondence to John J. Mullins, Centre for Genome Research, The University of Edinburgh, King's Buildings, West Mains Road, Edinburgh EH9 3JQ, United Kingdom. Phone: 44-131-650-6846; FAX: 44-131-667-0164.

1. *Abbreviations used in this paper:* DAF, decay accelerating factor; ES, embryonic stem; HAR, hyperacute rejection; ICM, inner cell mass.

morula/blastocyst stage in recipient rabbits or sheep, enables sexing, transgene screening, and cloning to take place before reintroduction into the natural host, providing that such screening methods are robust and reliable.

The major problem regarding pronuclear microinjection is that the exogenous DNA integrates randomly into chromosomal DNA. Position effects, where the transgene is influenced by its site of integration in the host chromosome (5), can have major consequences on the expression of the transgene, including loss of cell specificity, inappropriately high copy number–independent expression and complete silencing of the transgene. This is of greater concern in nonmurine transgenesis where the investment is higher. Position-independent, copy number–related expression can be achieved using sequences such as the locus control regions identified upstream of the β-globin gene cluster and downstream of the CD2 gene (6, 7), the A elements which flank the chicken lysozyme gene (8), and matrix attachment regions (9). Such elements have been shown to function across species barriers, and their incorporation into gene constructs can overcome position effects and improve expression of heterologous genes within specific cell types (5). In many cases, simply including large amounts of flanking sequences may be sufficient to overcome position effects and direct expression to specific tissues. To this end, the development and use of P1 (10), bacterial artificial chromosome (BAC) (11) and yeast artificial chromosome (YAC) vectors (12) for cloning of large segments of DNA, should greatly improve the chances of including important regulatory elements, including those involved in chromatin structure, within the transgene construct.

Embryonic stem cell technology

With the development of ES cell technology in the mouse (2), genetic manipulations can be performed in cell culture using appropriate selection strategies to permit the directed integration of the transgene to a specific region of the chromosome via homologous recombination. With the advent of homologous recombination, the researcher is able to insertionally inactivate, replace, or introduce subtle alterations to the endogenous gene of interest. Once the intended genetic change has been verified, the appropriate ES cells are introduced into blastocysts by microinjection, and, during subsequent gestation, may contribute to the developing embryo. If such a contribution is made, then by definition the resulting animal would be chimeric, being derived in part from the ES cells originating in culture. Assuming that the chimerism extends to the germline, then an appropriate breeding strategy will lead to the recovery of nonchimeric heterozygotes and, if viable, mice which are homozygous for the genetic change.

Most attempts to isolate and culture inner cell mass (ICM) cells from other species are based on the methods used for the

mouse. ES cells are maintained in culture in the presence of mouse-derived differentiation-inhibiting agents, provided either as a media supplement or through cocultivation in the presence of feeder cells. It has been suggested that these mouse-derived agents do not adequately prevent differentiation of stem cells in species other than the mouse, and pluripotent rat ES cells, capable of producing chimeras, were found to grow best on primary rat embryonic fibroblasts as the feeder layer (13). Freshly isolated cells from ICMs have been injected into blastocysts to produce chimeric offspring in both sheep and cattle (14), and their totipotency at this stage is further demonstrated by their ability to produce offspring after transfer into enucleated oocytes (15). Such nuclear transfer techniques are potentially very useful for the production of clonal offspring and would avoid the initial chimeric generation necessitated by the injection of ES cells into blastocysts. Recently, bovine-specific culture methods have shown promise with cells of up to 27 d of age maintaining their ability to direct normal calf development following nuclear transfer (16). However, at the present time the reliable generation of bovine ES cell lines requires the pooling of ICMs from several blastocysts and further efforts are required to enable the long-term culture of clonal bovine ES cells. Although to date chimeric animals have been generated from several species including the pig (17), in no species other than the mouse has germline transmission of an ES cell been successfully demonstrated. This remains a major goal for the future and may well require the use of novel strategies which depart widely from the traditional methods used in the mouse.

Nonmurine species in biomedical research

Selected physiological questions may be more conveniently modelled in the rat or in larger species. Not only can physical size be an advantage for biochemical sampling and physiological analyses, but certain genes may provide useful information when introduced into, for example, the rat genome when parallel experiments in the mouse would be ineffective. Examples include the modulation of blood pressure by the mouse *Ren-2* gene (18) and the modeling of inflammatory disease (19). In both cases, but for different reasons, no phenotype was observed in the respective transgenic mice, highlighting one of the advantages of having alternative species for understanding physiological mechanisms and the etiology of disease. More recently, a number of transgenic experiments have been undertaken to investigate lipoprotein metabolism. The human apolipoprotein A-1 gene was successfully expressed in the rat (20), resulting in increased serum HDL cholesterol concentrations, and attempts to therapeutically lower apo B100, and hence LDL and lipoprotein(a) concentrations, in the rabbit were successful (21) but resulted in complications. Although the targeted expression of the apo B–editing protein in the liver of the transgenic rabbits resulted in reduced LDL and lipoprotein(a) concentrations as intended, many of the animals developed liver dysplasia, suggesting that high level expression of the editing protein had unforeseen and detrimental side effects, possibly via the editing of other important mRNAs. The rabbit has also been used in HIV-1 research, with the development of a line expressing the human CD4 protein on T lymphocytes (22). Susceptibility to HIV infection was demonstrated, and although the rabbits are less sensitive to infection than humans, they may represent an inexpensive alternative to primates for many studies.

Gene transfer in farm animals was initially aimed towards improving production efficiency, carcass quality (23), and disease resistance of livestock. However, it has been suggested that the simple over-expression of hormones such as growth hormone may have unacceptable side effects. Recently some elegant studies of growth using transgenic rats have been performed and are likely to yield valuable information on the biochemistry and physiology of growth (24, 25). A more successful application of transgenesis in farm animals has been the production of biomedically important proteins. The two most popular methods have been to direct expression to hematopoietic cells or to the lactating mammary gland. In the former case, transgenic swine expressing high levels of human hemoglobin were generated using the locus control region from the β-globin gene cluster to overcome positional effects and direct expression to the hematopoietic cells (26). However, due to its natural ability to synthesize and secrete large amounts of protein, the mammary gland has become the primary focus for the expression of heterologous proteins in large mammals. Transgene expression has been successfully directed to the mammary gland using promoter sequences from milk protein genes such as those encoding ovine β-lactoglobulin (BLG), goat β-casein, and murine whey acidic protein. The BLG promoter was used to direct expression of human α_1-antitrypsin in lines of transgenic mice and sheep (27). Interestingly, a wide variation in expression was observed between mouse lines, and from one lactation to another within a single line. In sheep however, similar high levels of heterologous protein were expressed in milk over consecutive lactations and over several generations in a given transgenic line, allowing the viable development of a flock of transgenic sheep. In separate studies high levels of expression of human tissue plasminogen activator were obtained in goat's milk under the control of the goat β-casein promoter (28). The development of suitable purification methods and the use of transgenically produced proteins in clinical trials are well advanced, and, if successful, will have important implications for the production of human proteins in transgenic livestock. Poor expression of the ovine promoter in the mouse may reflect species differences in recognizing heterologous versus homologous promoters and raises questions concerning the predictive value of mouse models. At best therefore the generation of transgenic mice may, in certain cases, only be a guide to the potential success of a transgene construct in another species.

Gene transfer could equally be used to enhance the quality and suitability of milk derived from domesticated animals as a food for human consumption. Human milk is devoid of β-lactoglobulin, which is responsible for most of the allergies to cows' milk, and has a relatively high content of lactoferrin, which is important in iron transport and combating bacterial infections. One could envisage in the future the reduction of saturated fat content in cows' milk and the knock-out of unwanted proteins or their replacement with other more useful components. Through the manipulation of milk constituents it should be possible to more closely emulate the desirable components of human milk. The alteration of milk composition would appear to be a practical possibility given that milk micelles are remarkably tolerant to changes in composition, as demonstrated by the knock-out of the mouse β-casein gene (29). Ethical concerns regarding the generation of transgenic animals, which have been engineered specifically for pharmaceutical, medical, or nutritional reasons, lie outside the scope

of this overview, however it must be clearly ascertained that expression of a transgene does not compromise the animal.

Xenograft organs for transplantation surgery

The shortage of human organs for transplantation has raised interest in the possibility of xenotransplantation, i.e. the use of animal organs (30). However, the major barrier to successful xenogeneic organ transplantation is the phenomenon of complement-mediated hyperacute rejection (HAR), brought about by high levels of circulating natural antibodies that recognize carbohydrate determinants on the surface of xenogeneic cells. After transplantation of the donor organ, a massive inflammatory response ensues through activation of the classical complement cascade. This leads to activation and destruction of the vascular endothelial cells and, ultimately, the donor organ. The membrane-associated complement inhibitors, endogenous to the donor organ, are species restricted and thus confer only limited resistance. The complement cascade is regulated at specific points by proteins such as decay accelerating factor (DAF), membrane cofactor protein, and CD59. These regulators of complement activation are species specific. The initial strategy used to address HAR in porcine-to-primate xenotransplantation was to produce transgenic pigs expressing high levels of the human terminal complement inhibitor, hCD59. This was shown to protect the xenogeneic cells from human complement-mediated lysis in vitro (31). More recently, organ transplantation has been achieved using donor pigs which expressed human DAF on their endothelium (32), or both DAF and CD59 on erythrocytes, such that the proteins translocated to the cell membranes of endothelial cells (33). After transplantation, the pig hearts survived in recipient baboons for prolonged periods without rejection (33). Clearly, such genetic manipulations are bringing xenotransplantation ever closer to reality. If the isolation of suitable ES cells and application of homologous recombination becomes a reality in the pig, it may be possible to knockout the antigenic determinants to which antispecies antibodies bind, as a further strategy for eliminating HAR.

Summary

The use of nonmurine species for transgenesis will continue to reflect the suitability of a particular species for the specific questions being addressed, bearing in mind that a given construct may react very differently from one species to another. The application of transgenesis in the pig should produce major advances in the fields of transfusion and transplantation technology, while alterations in the composition of milk in a range of domesticated animals will have major effects on the production of pharmacologically important proteins and could eventually lead to the development of human milk substitutes. Despite the lack of germline transmission to date, major efforts continue to be directed towards the generation and use of ES cells from nonmurine species, using both traditional and new technologies, and the availability of such cells is likely to accelerate both the use of such species and the precision with which genetic changes can be introduced.

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Reproduction, Fertility and Development

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Progress and Emerging Problems in Livestock Transgenesis: a Summary Perspective

R. F. Seamark

*Department of Obstetrics and Gynaecology, The University of Adelaide,
Adelaide, S.A. 5005, Australia.*

Abstract. The creation of transgenic livestock is a complex multistep procedure the successful execution of which demands a high level of skill and application. Useful animals have been generated by transfer of genes to zygotes by microinjection, but further extension to livestock breeding is severely limited by the present low efficiency and lack of precision in gene transfer procedures. There are major developments in alternative approaches to gene transfer and those based on embryonic stem (ES) cell lines show particular promise as a broadly adaptable means of allowing precise manipulation of specific genes within the animal genome. Rapid progress is being made in adapting ES cell technology to livestock species but as yet no one has demonstrated the totipotency of the putative cell lines so far generated. The demonstration of the feasibility of the chimaeric route for reinstating an ES cell genome into the germ line of the pig is a major advance. For other livestock breeds, particularly those with long generation times and bearing single young where the chimaeric route is much less useful, there are encouraging developments in nucleus transfer (cloning) technology which could provide practical solutions. Overall, there are now good reasons to be optimistic that transgenesis will eventually be available to all livestock breeders with the proviso that there are no further unanticipated phenomena such as the effect of tissue culture on imprinting, to be discovered to threaten the predictability of outcome of ES cell-derived pregnancies and further limit the potential usefulness of this futuristic technology to the livestock industry.

Introduction

It is clear that transgenesis remains the most exciting and far reaching of recent advances in animal biotechnology. To the livestock breeder, the potential applications are legend and ordained to stretch the boundaries of human imagination.

It is also clearly evident that the term transgenesis implies more now than it did a decade ago when it was first coined by Gordon and Ruddle (1983). Then it was used to describe a technical process enabling transfer of inheritable, functioning genes between organisms, irrespective of species barriers. Now, following the advent of embryonic stem (ES) cell technology, it encompasses a process which allows the full armoury of recombinant DNA technology to be applied to enable molecular tinkering at all sites within the animal genome.

Although various studies have provided insight into what this new technology could offer to the livestock breeder, scientific and technical challenges still confront the molecular and reproductive biologist attempting to make the technology available to serve this purpose.

This paper briefly overviews the progress and emerging problems in livestock transgenesis in the light of the preceding discussion to provide a summary update.

Transgenesis: a Multistep Procedure

Transgenesis is a multistep procedure with each step testing the limits of advanced breeding knowledge and techniques. As such, the overall success rate is critically dependent on achieving the maximum efficiency for each of the individual steps. At present, no steps are 100% efficient and losses quickly compound resulting in the very low yields seen. Typically, by means of direct microinjection, purportedly the most efficient of the physical–chemical routes of gene transfer, only 1–2% of eggs injected result in transgenic young in livestock breeds. Furthermore, the transgenes insert at random sites within the genome to interrupt normal gene function on at least 10% of occasions. There is also frequent loss of the anticipated control of expression of the transgenes that are incorporated as a result of positional effects. Such inefficiency has major cost implications in livestock breeding and especially to cattle which produce single young and have a long generation time.

There have been various attempts to improve the efficiency of gene incorporation, by injecting oocytes and zygotes at different stages of the cell cycle (Powell *et al.* 1992) or by means of UV light (French *et al.* 1990), restriction enzymes, dyestuffs or other means

in association with microinjection to increase potential insertion sites within the host DNA; however, so far, there has been no significant improvement.

Embryo culture has proved helpful in allowing selection of viable embryos after injection (Powell et al. 1994) but reliable identification of pre-implantation embryos containing integrated transgenes through polymerase chain reaction (PCR), or other means, is proving elusive (Eyestone 1994). However, there now appear to be several promising ways of overcoming positional effects including: flanking injected transgenes with base sequences homologous to targeted insertion sites (Wilmut and Whitelaw 1994); the use of overlapping gene sequences (Clark et al. 1994); and the use of dicistronic targeting vectors to couple position-independent and position-dependent transgene sequences (Mountford et al. 1994). The proposed use ot precisely-located FLP recombinase target sites within the genome to allow both insertion and exchange of transgene cassettes through the FLP recombinase system at an efficiency comparable with the zygote microinjection approach is a particularly exciting development (Wigley et al. 1994).

The creation of separate artificial mammalian chromosomes (MACs) to contain the transgenes as described by Montoliu et al. (1994) would be the ultimate approach towards obtaining precise transgene control, but whether MACs can be made large enough to persist in the livestock genome without loss remains speculative. Of the alternative biological routes, viral vectors are potentially a 100% efficient means of achieving gene transfer but have specific limitations in the size of the gene construct that can be incorporated and problems of regulation owing to their uncontrolled site of insertion. Research exploring this approach is also heavily constrained by institutional regulatory bodies because of concerns with respect to the potential biohazardous nature of the vectors.

Sperm-mediated transfer would be a serious contender in livestock transgenesis if the efficiencies claimed by some groups (5%) (Lavitrano et al. 1989; Laura and Gandolfi 1993) could be more readily duplicated. The major appeal of this type of transfer is that, procedurally, it is easily incorporated into routine breeding systems and would allow a practical means of enhancing, in a directed way, the genetic potential of a herd or flock within current husbandry practices. One useful extension of this approach currently under investigation, is to couple improved methods of sperm-mediated transfer with in vitro maturation (IVM) and in vitro fertilization (IVF) procedures (Bachiller et al. 1991; Gagné et al. 1991). This would reduce the amount of DNA required and allow better regulatory control if transformed zygotes could be identified before transfer. There have been recent claims of successful extension of sperm-mediated transfer to a sheep transgenesis programme in Beijing, China,

(Y. F. Dai, personal communication) and a resurgence of interest in this approach can be anticipated.

Intravenous DNA delivery (Zhu et al. 1993) as a means of generating germ line transgenics remains speculative, but it could have immediate potential usefulness for production of pharmaceuticals in livestock.

ES Technology in Livestock: Current Progress

The exciting prospect of ES cell technology is that it can overcome the limitations relating to inefficiencies in gene transfer or manipulation by providing an abundance of totipotent ES cells required to allow genetic manipulation by conventional recombinant DNA techniques. A variety of procedures are now available to ensure that those cells transformed according to plan can be identified, cloned and maintained as isogenetic cell lines. The vision is that the modified ES cell genome can then be reinstated within the germ line, thus providing an almost inexhaustible source of identical, superior transgenic stock with revolutionary impact on the livestock industry.

There are now many encouraging reports which clearly indicate that pluripotent ES cells can be created for the major livestock breeds, most notably the pig (Wheeler 1994); however, as yet, no group has demonstrated totipotency of these cells through reinstating their genome within a germ line.

Furthermore, for reasons that any practitioner in this area of ES cell research would be aware of, even if such cell lines were already available, the brave new world envisaged for the livestock breeder could not be realized immediately as procedures for reinstating the ES cell genome into a germ line are still far from routine. Several routes are potentially available for this purpose. In the mouse, the most commonly used approach is to inject about 10–15 isolated ES cells into a blastocoel of a host blastocyst and to allow the cells to mix with the cells of the inner cell mass. The resultant chimaeric blastocysts are then transferred to recipients for rearing. In skilled hands, about 30% of the chimaeric offspring are germ line chimaeras capable of producing young with the ES cell genotype. Potentially, this figure can be increased through the use of very early (8–16-cell) stage embryos (Tokunaga and Tsunoda 1992) and tetraploid or otherwise handicapped blastocysts (Nagy et al. 1990), as hosts for ES cells.

As now convincingly shown by Wheeler (1994), this route can be adopted without major modification for putative pig ES cells provided one is persistent enough and chooses the right breeds. Their success also augurs well for the application of the chimaeric route to other livestock breeds, accepting the obvious constraint that a successful outcome, i.e. the creation of germ line chimaeras, can only be known in the following generation, a delay of 3–4 years in the case of cattle.

The alternative, more desired and, thus, preferred route towards reinstating the ES genome in the germ line which is now under active consideration by all major groups, is by means of nucleus transfer. The feasibility of this approach for livestock species was first indicated by Smith and Wilmut (1989) when they successfully created viable sheep zygotes by fusing individual inner cell mass cells with enucleated oocytes. This route is preferred as, when applied to ES cells, it ensures that all the cells in the offspring, including the germ cells, are of the ES cell genotype. The technical approach presently favoured by researchers remains firmly based on the cloning procedures pioneered by Willadsen (1986) where nucleus transfer is achieved by electrofusing a karyoplast with a surgically enucleated oocyte (cytoplast) derived from in vivo or in vitro sources. However, when used in cloning 16–64-cell stage embryos, the overall success of this process is only 6–7 lambs or calves born per 100 karyoplasts (blastomeres) fused and success with the smaller inner cell mass cells or ES cells may be expected to be less. Within reasonable limits, this low efficiency would be negated by the abundance of ES cells available from a given cell line and the unlimited supply of oocytes from an abattoir source that could be generated by means of IVM procedures. Simple non-surgical methods of enucleating oocytes are also under development to facilitate this approach (Fulka and Moor 1993; Tatham et al. 1993). However, it may be anticipated that the maintenance of high overall yields in a complex multistep procedure such as nucleus transfer will remain a significant technical challenge to the embryologist. In the improvement of efficiency, one area of active research relates to the need to develop simple procedures to ensure a higher yield of zygotes through the matching of the stage of cell cycle of recipient oocytes and nucleus donor cells. Concomitantly, new ways of achieving cell fusion need to be devised as electrofusion often activates oocytes inappropriately owing to the major changes in cellular ion flux it induces. As a consequence, there is renewed interest in the use of Sendai virus (Tsunoda and Kato 1993) and chemical fusagens as alternative routes to nucleus transfer. However, as yet, no procedures have been developed for achieving nucleus transfer and activating the oocyte as subtle as those used by the sperm at fertilization. The isolation and sequencing of the fusagens and the active principle(s) contained within the sperm head which evoke the normal activation response of the egg would be a major advance in this area.

Embryo culture is widely used in nucleus transfer programmes as a means of selecting purportedly viable zygotes suitable for transfer and thus minimizing the number of recipients needed. Most commonly, this entails transfer to the Fallopian tube of a temporary host, usually a sheep or a rabbit. A period of in vitro culture would be more convenient for this purpose but the ideal choice of media and culture conditions required for this purpose is still far from resolved (Trounson et al. 1994). The simple culture media, favoured by several groups including this laboratory, is now known to have long-term adverse impact on embryo viability (Walker et al. 1992) and the usefulness of co-culture and more complex media remains to be fully evaluated. In part, the lack of resolution in this area can be ascribed to a reluctance by many researchers to accept that ultimate proof of new culture procedures does require embryo transfer and assessment of the viability of young. The various claims for many of the cell fusion procedures now extant are still based mainly on the numbers of blastocysts generated in culture, despite the increasing evidence of significant loss of these blastocysts after transfer. It remains to be determined to what extent such losses relate to the nucleus transfer process itself, or are artifacts of the various culture systems.

ES Technology in Livestock: Emerging Problems

There is now growing appreciation that development of the preimplantation embryo reflects not only the genomic contribution of both parents but the quality of the environment to which the embryo is exposed.

Even small changes in this environment can impact on development with long-term implications on health and welfare. For example, in the mouse, brief exposure of preimplantation embryos to in vivo culture conditions can both result in substantial phenotypic variation and predicate the subsequent expression and penetration of some transgenes (Kothary et al. 1992 and reviewed by De-Groot and Hochberg 1993). In the same, an extended period of embryo culture (3–5 days) can result in dramatically enhanced peri- and post-natal loss and abnormally large lambs (Walker et al. 1992).

Asynchrony between the stage of development of the embryo and tract at embryo transfer can also affect development as first indicated by Wilmut and Sales (1981) in the ewe; this was also recently substantiated by Kleeman et al. (1994) who found that fetal growth was enhanced abnormally by advancing the stage of development of the tract in recipient ewes through administering progesterone during the first three days of pregnancy. Similar results had been previously obtained in the cow (Garrett et al. 1988). Further study of the full range of epi-genetic factors influencing gene expression during this critical developmental period is thus clearly warranted.

Insight into the cellular and molecular biology underlying these phenomena has been provided by studies characterizing the tract in terms of the production of growth factor and cytokines and the embryo in terms of its receptors (Gandolfi 1994; Watson et al. 1992, 1994). These studies, together with others on the impact of

macromolecules (Hill *et al.* 1994), redox active substances and various substrates (Trounson *et al.* 1994), clearly indicate both the complexity and subtlety of the tubal and uterine environment and the communication networks influencing the development of the conceptus.

The contingencies imposed by culture and other embryo micromanipulation procedures used in ES cell culture and nucleus transfer on imprinted genes such as insulin-like growth factor 2 (IGF2) and its receptor (IGF2r) may provide particular insight in relation to anomalies in fetal growth and development, as would the study of cytokines and other growth factors which interact directly with the genome. Culture conditions are also known to alter the status of imprinted genes within ES cell lines and to change both the genotype and phenotype of cell lines in culture (Parchment and Natarayan 1992) with unanticipated implications for procedural outcomes Pedersen 1994). The degree to which these or other wild cards yet to be discovered will curtail the eventual usefulness of transgenic ES cells to the livestock breeder remains a matter of speculation.

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